NEUROTENSIN ENGAGES MESOLIMBIC DOPAMINE CIRCUITS TO REGULATE BODY WEIGHT

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Physiology—Doctor of Philosophy

2017

ABSTRACT

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Body weight is determined by feeding and volitional physical activity behaviors that are regulated, in part, by dopamine (DA) neurons of the ventral tegmental area (VTA). Here, we sought to understand how the neuropeptide, neurotensin (Nts) engages VTA DA neurons to modify body weight. The rationale for this work is that pharmacologic application of Nts into the VTA suppresses food intake and promotes locomotor activity, yet the endogenous circuits by which Nts acts on the VTA to modify these behaviors and body weight remain unclear. First, we identified the endogenous sources of Nts input to the VTA; using retrograde tracing we found that the lateral hypothalamic area (LHA), a critical neural hub for coordinating energy balance, provides substantial Nts projections to the VTA. We next examined how Nts directly engages VTA DA neurons by identifying Nts receptor-expressing cells in the VTA. To do this, we generated mice expressing Cre-recombinase in Nts receptor 1 (NtsR1) or Nts receptor 2 (NtsR2) cells, which revealed that NtsR1 is expressed on many VTA DA neurons, whereas NtsR2 is predominantly restricted to glial cells. Furthermore, only the VTA NtsR1 neurons project to the nucleus accumbens (NA), where DA release is known to modify feeding and locomotor behavior. We therefore tested the physiologic necessity for Nts action via the VTA by genetically ablating VTA NtsR1 neurons. Mice lacking VTA NtsR1-DA neurons were hyperactive, failed to gain weight, and could not appropriately coordinate feeding behavior with peripheral energy cues, demonstrating that VTA NtsR1 neurons are essential for energy balance. Finally, we tested the hypothesis that endogenous Nts input from the LHA to the mesolimbic DA system would be sufficient to regulate body weight. Indeed, chemogenetic activation of LHA Nts neurons increased physical activity, restrained food intake, and promoted

weight loss in lean mice. Interestingly, the anorectic effects of LHA Nts activation were mediated via NtsR1 and DA signaling, while the physical activity was NtsR1-independent. Furthermore, in hungry mice (a state in which increased appetitive drive can promote overeating and weight gain), activation of LHA Nts neurons suppressed intake of chow and palatable sucrose rewards. Collectively, this work defines an endogenous LHA Nts circuit that engages the mesolimbic DA system via NtsR1 to suppress food intake in both energy replete and energy depleted states. Enhancing action via this circuit may thus be useful to support dual weight loss behaviors in an obesogenic environment.

To my family, labmates, and mentors

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Gina Leinninger for her unremitting support, encouragement and enthusiasm over the last five years. Not only are you my mentor, but you are also an authentic role model, leader, and teacher whom I admire for your communication skills and respect for others. Thank you for showing me how much fun science can be, for being my advocate, and for always helping me feel like my work was valued. It's been an honor and a privilege to be your graduate student.

I also want to thank Dr. Steve Heidemann, a long-time mentor whom I first met as an undergraduate and who connected me to Gina Leinninger. Your kind, sage, and honest advice helped guide my career trajectory toward the right track on multiple occasions and I can't express how much I appreciate it. I would like to thank Drs. Michelle Mazei-Robison and A.J. Robison for always having open doors and being willing to help me with experiments and give advice. I would also like to thank my thesis committee members, Drs. Michelle Mazei-Robison, Cheryl Sisk, Alex Johnson, and Bob Wiseman for helpful suggestions, discussion, and support.

I am indebted to three talented undergraduate students who made substantial contributions to the project: Hannah Batchelor, Trevor Lewis and Bethany Beekly. Thank you for your willingness to learn, constant curiosity, and attention to detail. Your hard work made a huge difference and I couldn't be more proud to have been your mentor. I would like to thank all the members of the Leinninger lab—Raluca Bugescu, Juliette Brown, Gizem Kurt, Patricia Perez-Bonilla, and Laura Schroeder, for being my support system and treating me to some of the best philosophical conversations with many laughs over food and drinks. Much of this work would not have been possible without Sandra O'Reilly—I truly appreciate how consistent and thorough you were with my metabolism experiments. Many members of the Mazei-Robison and Robison labs helped me on numerous occasions with behavioral experiments, particularly

Andrew Eagle and Ken Moon. I am so grateful for the hours you spent helping me set up equipment, answering my questions, and looking at my data.

None of this work would have possible without the animal care staff—thank you for taking such good care of our mice. I'd also like to thank Susanne Mohr and Cindy Arvidson for providing me with support and guidance as a student in the Physiology PhD Program and MSU MD/PhD Program. Finally, I would to express my gratitude for the entire Physiology faculty and staff who have been continuously helpful, friendly, and interested. Thank you for making me feel at home for the past five years.

This work was supported by the NIDDK: HLW (F30-DK107163) and GML (R01-DK103808).

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KEY TO ABBREVIATIONS

3N	oculomotor nucleus
3V	third ventricle
4V	fourth ventricle
aca	anterior commissure
AHA	anterior hypothalamic area
APTN	anterior pretectal nucleus
aq	cerebral aqueduct
ARC	arcuate nucleus
ATg	anterior tegmental nucleus
BLA	basolateral amygdala
cc	corpus callosum
CeA	central amygdala
ChR	channel rhodopsin
CNO	clozapine-N-oxide
cp	cerebral peduncle
CPP	conditioned place preference
CPu	caudate/putamen
D1R	dopamine receptor 1
D2R	dopamine receptor 2
D3V	dorsal third ventricle
DA	dopamine
DAT	dopamine transporter
DMH	dorsomedial hypothalamus
DR	dorsal raphe
DREADD	Designer Receptors Exclusively Activated by Designer Drugs

DTg	dorsal tegmental nucleus
EA	extended amygdala
ec	external capsule
En	endopiriform cortex
f	fornix
FG	fluorogold
fr	fasciculus retroflexus
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acid protein
GFP	green fluorescent protein
GP	globus pallidus
ic	internal capsule
ICV	intracerebroventricular
IL	infralimbic cortex
ip	interpeduncular nucleus
i.p.	intraperitoneal
IPAC	interstitial nucleus of posterior limb of anterior commissure
ISH	*in situ* hybridization
LDTg	laterodorsal tegmental nucleus
LepRb	leptin receptor
LHA	lateral hypothalamic area
LHb	lateral habenula
LO	lateral orbital cortex
LPO	lateral preoptic area
LS	lateral septum
lv	lateral ventricle

MCH	melanin-concentrating hormone
MCR-4	melanocortin receptor-4
MHb	medial habenula
ml	medial lemniscus
MPN	medial preoptic nucleus
MPO	medial preoptic area
MS	medial septum
α-MSH	alpha-melanocyte stimulating hormone
mt	mammillothalamic tract
NAc	nucleus accumbens core
NAsh	nucleus accumbens shell
ns	nigrostriatal tract
Nts	neurotensin
NtsR1	neurotensin receptor 1
NtsR2	neurotensin receptor 2
OFT	olfactory tubercle
ot	optic tract
OX	orexin/hypocretin
PAG	periaqueductal gray
PBN	parabrachial nucleus
pc	posterior commissure
PFC	prefrontal cortex
POA	preoptic area
PPTg	pedunculopontine tegmental nucleus
PrL	prelimbic cortex
pSTN	para subthalamic nucleus

PVH	paraventricular hypothalamus
RER	resting energy expenditure
RMTg	rostromedial tegmental nucleus
RLi	raphe linear nucleus
SC	superior colliculus
scp	superior cerebral peduncle
SN	substantia nigra
SPTg	subpedunculuar tegmental nucleus
ST	stria terminalis
STN	subthalamic nucleus
TEA	thermic effect of activity
TEF	thermic effect of food
TH	tyrosine hydroxylase
ts	tectospinal tract
VMH	ventromedial hypothalamus
VP	ventral pallidum
VTA	ventral tegmental area
ZI	zona incerta
xscp	decussation of superior cerebellar peduncle

Chapter 1. A Weighty Introduction: Neurotensin and Dopamine Circuits that Regulate Energy Balance

1.1 Background and Significance

Global rates of obesity have grown significantly over the past three decades with >1.9 billion adults qualifying as overweight or obese in 2015[1,2]. Obesity is a particularly serious problem in the U.S., where almost 70% of adults have a BMI in the overweight or obese category (>25 kg/m^2) and minority populations are disproportionately affected[3]. U.S. childhood obesity rates have also sky-rocketed, with 20% of children ages 6-19 qualifying as obese nation-wide[4,5] and rates upwards of 32% are present in certain locations such as the South Bronx[6]. Obese children are 80% more likely to be obese as adults and have increased risk of early death[7], and in fact, increased body mass has now surpassed undernutrition as a top cause of death and disability worldwide[8]. Furthermore, being overweight increases risk for developing chronic conditions such as type-2 diabetes, heart disease, stroke, cancer, fatty liver disease, and kidney disease, conditions that are expensive to treat and reduce quality of life[9,10]. The high incidence of obesity already exerts a considerable toll on healthcare systems worldwide, but the rising costs associated with treating obesity/overweight and concomitant complications will be unsustainable without intervention[11]. Curtailing the obesity pandemic is thus a global health concern of paramount importance.

Despite the overwhelming prevalence of obesity and overweight, few medical strategies to date have proven effective in maintaining long-term weight loss. The first-line prescription for weight reduction is diet and exercise, which has been capitalized by the U.S. weight loss industry, a market worth $60 billion in 2015[12]. While many individuals initially lose weight through dieting, most do not maintain the weight loss long-term[13]. A regimen of both diet and

exercise is more likely to provide long-term benefit[13], however adherence to such lifestyle modification is notoriously challenging. Given the high likelihood of weight regain, many dieters enter a vicious cycle of weight cycling or "yo-yo dieting" which increases risk of heart disease, stroke, and diabetes[14]. Thus, not only are lifestyle modifications largely ineffective, but repeated dieting and weight cycling imposes adverse health effects. A handful of pharmacologic agents have been developed to aid weight loss, but most have side effects and not all are approved for long-term use[15]. The effectiveness of obesity medications is typically modest, with most agents achieving 5% loss of body weight at 1 year[16,17]. While 5% weight loss can reduce risk of obesity-related complications, it is not enough to achieve a healthy BMI for most patients. Additionally, few studies have examined outcomes including potential weight regain after patients stop taking the medication, thus the long-term effectiveness of anti-obesity drugs is largely undetermined. Currently, the most effective obesity treatment is bariatric surgery, with the Roux-en-Y gastric bypass procedure (RYGB) producing an average loss of 5 BMI points at 5 years[18,19]. In addition to substantial weight loss, bariatric surgery also alleviates obesity-related type 2 diabetes, hypertension, and joint pain[18], plus patients report increased quality of life after surgery[20]. The major drawbacks of bariatric surgery include the risk of complications and the cost which averages $15,000-$25,000[21,22]. Due to this, surgery is usually reserved as a last resort for the morbidly obese (BMI>40 kg/m^2), which comprise less than 10% of overweight or obese adults. Thus, moderately overweight or obese individuals who are still at risk for developing obesity-related complications have limited options for weight control (diet and exercise or pharmacotherapy), which provide only modest benefit. Therefore, there is a clear need to develop better strategies to promote weight loss and prevent weight gain to improve health outcomes for overweight and obese individuals.

It is also vital to understand why the surge in overweight and obesity has occurred, as this may inform design of interventions to manage body weight. Average caloric intake has

increased by around 240 kcals/day since 1970, with most of the increase attributed to carbohydrates[23]. Interestingly, fat intake decreased over the same time period as obesity rates continued to rise, suggesting that excess caloric intake, not high dietary fat consumption, potentiates weight gain. Further, occupational-associated energy expenditure has progressively declined since 1960[24] and only 1 in 5 adults fulfills the recommend amount of daily physical activity[25]. Thus, one would speculate that the obesity epidemic is fueled by excess caloric intake combined with reduced physical activity. While this certainly explains what causes obesity, it does not explain *why* individuals overeat and move less. The increasing availability of palatable, calorie-rich, and inexpensive food has fueled obesity rates[26], but what permits caloric intake in excess of metabolic demands? Under normal circumstances, energy intake is exquisitely coordinated with energy expenditure in an effort to defend body weight from both loss and gain[27,28]. However, these mechanisms are not completely understood and are impacted by numerous variables (i.e. food palatability, genetics, sedentary lifestyle) that contribute to the development of obesity. The brain plays an essential role in coordinating motivated behaviors such as feeding and voluntary physical activity, but the neural circuits involved remain poorly understood. The purpose of this chapter is thus to provide an overview of energy balance physiology and describe how disruption of dopamine-mediated feeding and physical activity behaviors contributes to the development of obesity. We will also evaluate how the neuropeptide, neurotensin (Nts) may engage dopamine circuits, and hence the potential of the Nts signaling system as a novel target for weight control.

1.2 Coordination of Energy Balance

1.2.1 What is Energy Balance and How Does it Determine Weight?

The term “energy balance” is used to describe the relationship between energy intake and expenditure that determines body weight. Energy intake consists of calories consumed

through food and liquids. Energy expenditure refers to the calories burned to support basal metabolism and voluntary physical activity. For most individuals, energy expenditure is the sum of resting metabolic rate (RMR), the thermic effect of feeding (TEF), and the thermic effect of activity (TEA)[29]. RMR comprises 60-75% of total energy expenditure and is the energy required by the body to perform basic physiologic functions, or more simply, "the number of calories an individual would use if he/she stayed in bed all day." TEF accounts for 10% of energy expenditure and is the energy required for digesting food. TEA can account for 15-30% of energy expenditure and refers to the additional calories burned through volitional activity and exercise[29]. When energy intake exceeds expenditure it creates a caloric surfeit, or "positive energy balance", which can be stored in the body as fat and lead to weight gain. Conversely, when energy expenditure exceeds caloric intake, the body experiences a caloric deficit or "negative energy balance"; as a result, calories required to support survival are obtained from adipose reserves, leading to weight loss. At face value, energy balance appears to be a simple math equation, but its coordination is complex, requiring continuous communication between the periphery (to sense energy status) and the brain (to appropriately modulate energy intake and expenditure).

Energy balance is intimately tied to the idea of a body weight "set-point" wherein genetic and environmental factors determine an individual's body weight, which is defended through homeostatic mechanisms that compensate for positive or negative energy balance[28]. For example, in controlled over-feeding studies, total energy expenditure increases and appetite decreases as the body attempts to deplete the caloric surplus[30,31]. Similarly, weight loss leads to increased appetite and reduced energy expenditure that drives recovery of lost body weight and indeed, most people who lose weight gain it back[32-36]. Cota *et al.* provide a salient example of just how tightly the body coordinates energy balance: the average adult male uses around 900,000 calories per year and only gains (on average) one pound or 3600 extra calories during

that time, which amazingly amounts to >99% accuracy in matching food intake with energy expenditure[27]. Although energy balance is exquisitely fine-tuned in the short-term, small, incremental weight gain over years is thought to contribute to obesity. As such, the body weight set-point slowly drifts upward with time[28], and losing weight after being overweight or obese for several years is extremely challenging as the body strives to defend a heavier set-point[35,37]. A prime example of this physiology comes from study of obese participants on the TV show "The Biggest Loser": contestants lost >120 pounds on average during the show, but 6 years later, individuals had regained approximately 2/3 of the weight and their energy-expenditure was significantly decreased compared to what would be expected for their body weight[38]. Thus overweight individuals who have lost weight are constantly battling increased hunger and reduced energy expenditure as their bodies defend their heavier weight set-point.

1.2.2 The Brain Coordinates Energy Balance

Energy balance strongly relies on behavioral output, namely feeding and volitional activity that are controlled by the brain. Interestingly, most genes implicated in obesity have enriched expression in the nervous system[39], supporting the role of the brain as a master regulator of body weight. In particular, sub-regions of the hypothalamus have been implicated in regulating energy balance, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH) and the lateral hypothalamic area (LHA). The hypothalamus is found at the base of the brain near the third ventricle and is well positioned to intercept circulating energy cues from the periphery. Important hormonal cues detected by the hypothalamus include the anorectic hormone leptin and the orexigenic hormone ghrelin. Leptin is secreted in proportion to adipose tissue as a signal of long-term energy storage and acts on hypothalamic nuclei to suppress food intake[40]. Ghrelin, by contrast, is secreted from the stomach as hunger increases and acts on the hypothalamus and other brain areas to promote food intake[41]. The ARC is a key site for energy integration and has been extensively studied.

The ARC contains two discrete neuronal populations expressing either agouti-related peptide (AgRP) or promelanocortin (POMC), which exert opposing actions on feeding and body weight[42]. AgRP neurons are activated by physiologic hunger and promote food intake while reducing energy expenditure[43,44]. AgRP neurons also express neuropeptide Y (NPY) and GABA, and the contribution of each individual neurotransmitter has been shown to increase feeding[45]. AgRP neurons are active in a fasted state, which is potentiated by ghrelin-mediated excitatory input[46,47]. After a meal, ghrelin levels fall and leptin becomes a dominant circulating signal of energy status, which inhibits AgRP neurons to reduce food intake[48].

By contrast, neighboring POMC neurons are activated during satiation and suppress food intake while increasing energy expenditure[49-51]. POMC is a precursor protein that is cleaved into alpha melanocyte stimulating hormone (α-MSH) and exerts anorectic effects by binding the melanocortin-4 (MCR-4) receptors at key brain sites. Rodents deficient in MCR-4 signaling are hyperphagic and obese[52,53] and MCR-4 mutations are the most common monogenic cause of human obesity[54,55], underscoring the importance of melanocortin signaling in energy balance. While the ARC is a critical center for direct sensing of peripheral energy cues, POMC and AgRP neurons likely feed into a variety of downstream circuits that fine-tune feeding behavior and energy expenditure such as the lateral hypothalamic area (LHA). Indeed, POMC neurons project heavily to the LHA which expresses MCR-4[51,56], but the functional role of melanocortin signaling in the LHA has not been characterized. For the purposes of this chapter, we will focus on the LHA as a hypothalamic center that plays an essential, yet incompletely understood role in energy balance by both receiving input from the ARC and directly sensing peripheral energy cues.

1.2.3 The Lateral Hypothalamic Area (LHA) is Essential for Energy Balance

The LHA was initially described as a "feeding center" when investigators found that electrical stimulation induced voracious feeding behavior in sated animals[57,58]. Conversely, lesions of the LHA produced such profound self-induced starvation that animals had to be force-fed to maintain survival[58-60]. Subsequent neuroanatomical studies revealed that the LHA provides efferents to numerous brain regions, but it provides particularly dense projections to dopamine (DA) neurons in the ventral tegmental area (VTA) that modify the motivation to obtain pharmacological and natural rewards (e.g. food, physical activity, and sex)[61]. Indeed, electrical stimulation of the LHA is rewarding and increases DA release in nucleus accumbens (NA)[57,62-64]. Thus, the LHA directly accesses DA-ergic circuitry that controls motivated behaviors, and provides an explanation for why lesion of the LHA abolishes the motivation to eat. Additionally, the LHA receives information concerning energy status that may be important for appropriately coordinating feeding and other motivated behaviors. Some LHA neurons express receptors for leptin while others respond to ghrelin[65,66], indicating that the LHA can directly intercept circulating anorectic and orexigenic cues. The LHA also receives dense input from the ARC[51], and thereby receives indirect information regarding peripheral energy status. Taken together, this work suggests that the LHA is uniquely positioned to integrate peripheral energy cues with DA-mediated motivated behaviors that may promote or suppress feeding. Given that the LHA responds to both anorectic and orexigenic cues, there are likely distinct neural mechanisms by which the LHA can coordinate motivated behaviors and energy balance. Indeed, several populations of neurons have been described in the LHA that vary in neurotransmitter and neuropeptide content, projection targets, and function[67,68]. The contributions of the key neuropeptide-defined neuronal populations in the LHA with respect to energy balance will be briefly discussed below.

Orexin: Many neurons in the LHA express the orexigenic neuropeptide hypocretin/orexin (OX)[69]. As its name implies, OX stimulates food intake but also plays a critical role in wakefulness and arousal[68,70]. As such, DREADD-mediated activation of OX neurons increases feeding, locomotor activity, and energy expenditure[71]. OX neurons are activated by cues of energy depletion including fasting, ghrelin, and low glucose[66,72,73] and thus act to promote food intake and arousal necessary to obtain food when peripheral energy stores are low. Although OX-expressing neurons are only found within in the LHA, they project widely throughout the brain, including to the VTA[74,75]. OX neurons also co-express markers of glutamatergic neurons[76] and therefore can excite afferent projection targets such as VTA DA neurons[75].

Melanin-concentrating hormone: A separate subset of LHA neurons contains melanin-concentrating hormone (MCH), which similar to OX, promotes food intake and weight gain[77,78]. Instead of stimulating arousal however, MCH supports sleeping behavior[79]. MCH neurons express both glutamatergic and GABAergic markers, and thus may be divided into further functional subsets based on excitatory vs. inhibitory potential[79-81]. While MCH neurons do not project directly do the VTA, many instead project directly to GABA-ergic neurons in the NA that provide tonic inhibitory input on food intake[82-84]. Thus GABA-ergic MCH neurons may dis-inhibit the NA, removing the “brake” on feeding to promote food intake[67].

Neurotensin: A third molecularly distinct population of LHA neurons expresses the neuropeptide, neurotensin (Nts)[66,85]. Unlike the orexigenic effects of MCH and OX, pharmacologic Nts suppresses feeding and promotes weight loss[86-90], however the functional role of LHA Nts neurons has not been completely defined. A subset of LHA Nts neurons expresses the long form of the leptin receptor, are activated by leptin, and co-express GABA[65,85]. Some LHA Nts neurons project locally to and inhibit neighboring OX neurons[91] while

others project to the VTA[66,85], where pharmacologic Nts administration produces anorexia and locomotor activity[86,88,92]. Consistent with this, developmental loss of leptin receptor signaling specifically from LHA Nts neurons results in reduced striatal DA action, increased adiposity, and disrupted feeding response to leptin[66,85]. Nts is an established modulator of DA signaling (see section 1.4.1) and the LHA may provide an endogenous source of Nts input to the VTA that impacts motivated behaviors. In support of this, it was recently demonstrated that chemogenetic activation of LHA Nts neurons increased mesolimbic DA release, locomotor activity, and reduced body weight[93]. Thus, Nts and DA action may be useful to promote weight loss behaviors, while disruption of Nts-mediated DA signaling might contribute to the development of obesity. Understanding how Nts engages the DA system to modify motivated behaviors, including the specific role of LHA Nts neurons, will be important for discerning the therapeutic potential for Nts in regulating energy balance.

1.3 Dopamine Signaling and Body Weight

1.3.1 Dopamine Circuitry, Signaling, and General Function

Dopamine (DA) is a catecholamine neurotransmitter that is essential for movement and motivation[94], and DA disruption is a hallmark of several neuropsychiatric diseases. Neurons that synthesize DA are found primarily in the VTA and substantia nigra pars compacta (SN) of the midbrain. DA synthesis begins with the amino acid, tyrosine, which is converted to L-DOPA by tyrosine hydroxylase (TH), the rate-limiting step in DA synthesis. L-DOPA is rapidly converted to DA by DOPA-decarboxylase, and DA is stored in highly concentrated vesicles until an action potential initiates their release[95]. DA binds to five receptor isoforms, however most work has focused on the DA receptor 1 and 2 isoforms (D1R and D2R), both of which are expressed on GABA-ergic medium spiny neurons (MSNs) in the NA, and all are coupled to G-

proteins. D1R is coupled to G_{α} which increases protein kinase A (PKA) activity through induction of adenyl cyclase and cyclic adenosine monophosphate (cAMP), while D2R is coupled to G_i which essentially has the opposite effect and inhibits PKA activity[96-98]. PKA regulates a variety of downstream targets including AMPA and NMDA receptors, CREB, and DARP-32[99], and the outcome of DA receptor signaling therefore includes changes in glutamatergic regulation, ion channel phosphorylation, and gene transcription[100]. Interestingly, D2R is also expressed presynaptically on DA neuron terminals and dendrites and acts as an inhibitory autoreceptor that decreases DA synthesis and release[101-103]. After DA is released into the synaptic cleft and binds to DA receptors, it is taken back up into the pre-synaptic terminal by the DA transporter (DAT), where it can then be recycled back into vesicles or degraded (see[104,105] for review). VTA DA neurons project to the ventral striatum and prefrontal cortex (the mesolimbic and mesocortical pathways, respectively), whereas SN DA neurons project to the caudate and putamen (CPu), collectively referred to as the dorsal striatum (the nigrostriatal DA pathway). Generally speaking, the mesolimbic circuit controls motivation, while the nigrostriatal circuit regulates movement, hence the degeneration of nigrostriatal DA neurons in Parkinson's disease causes loss of motor function. While the VTA and SN may exert some overlapping contributions to motivation and movement[106], we will focus on role of VTA mesolimbic signaling in energy balance because it is implicated in regulating motivated feeding and locomotor behaviors that alter body weight.

DA has often been suggested to signify *reward*, however some have argued that this term is ambiguous and obfuscates the actual role of DA in behavior[107-110]. According to Berridge and Kringelbach, reward is a complex term that encompasses aspects of pleasure, reinforcement, and learning, all processes that are mediated by distinct neural circuits that may or may not involve DA. They suggest that procurement and consumption of rewards, such as food, sex or drugs, are mediated by at least two distinct neural mechanisms which are

described in terms of “liking” and “wanting”. “Liking” is analogous to the positive feeling or pleasure evoked during physical contact with the reward, while “wanting” refers to behavioral investment required to attain the reward. Although DA has been popularized as the “pleasure” neurotransmitter, pioneering work by Berridge *et al.* called this into question when they showed that DA-depleted rats have intact hedonic or “pleasure” responses to sweet taste[111]. This suggests that DA is not necessary for the “liking” of rewards, and has since been supported by further work showing that hedonic reactions are not impacted by interruption of DA signaling[109,110]. Instead, DA mediates the *effort* an animal is willing to exert to attain a reward, or “wanting.” Thus, DA depletion does not alter hedonic response to a reward, but it does reduce willingness to work for the reward or responsiveness to cues that predict the reward[112,113].

Salamone and Correa propose a similar model narrowed to mesolimbic DA release, whereby the behaviors necessary to approach the reward (analogous to “wanting”) require DA release in the NA, whereas the direct interaction with the reward (analogous to “liking”) is independent of NA DA[61]. Both the models of Berridge *et al.* and Salamone *et al.* align with the involvement of DA in reward prediction error, the observation that DA neurons fire based on the perceived expectations of how “rewarding” a particular stimulus will be. As described by Schultz, DA neurons fire in response to an unexpected reward and are inhibited when the reward is omitted[112]. In a sense, DA neurons code the “error” between perceived and actual expectations, thus explaining why disruption of DA signaling may interfere with the perceived value of a reward, not necessarily consumption of the reward itself[112,113].

A final note with respect to VTA DA neurons is that they are molecularly heterogeneous and can be divided into subsets based on their electrophysiological properties, projection sites, co-expression of classical neurotransmitters, and response to neuropeptide modulators[114]. While VTA DA neurons heavily project to the NA, they also project to the prefrontal cortex

(PFC), amygdala and hindbrain, and DA release in each of these areas plays unique roles in behavior[115]. For instance, VTA DA neurons projecting to the NA are activated by rewards and promote positive reinforcement, while VTA DA neurons projecting to the PFC are activated by aversive cues and promote conditioned-place aversion[116-118]. Thus, VTA DA signaling does not purely regulate pursuit of pleasant stimuli but is also processes aversive stimuli.

1.3.2 Dopamine and Feeding

The idea that DA is essential for movement and motivation came from early work showing that destruction of midbrain DA neurons leads to hypotonia and hypophagia[119,120]. Consistent with these observations, mice genetically lacking DA fail to gain weight and starve to death by 3 weeks of age unless DA is restored pharmacologically[94]. Since feeding requires physical movement, it was difficult to ascertain whether DA-deficient mice were hypophagic due to a primary feeding deficit or because of generalized impairment of motor function necessary to procure food. The latter explanation is supported by observations that animals with impaired DA signaling, either brain-wide or specifically to the dorsal striatum, ate less due to defective forepaw usage[121,122]. Furthermore, selective disruption of DA signaling in the NA does not impair free food intake, but instead reduces lever-pressing for food, which becomes more pronounced as the amount of work necessary to attain food increases[123-128]. These data suggest that NA DA release is not necessary for *ad libitum* feeding, but instead regulates the effort (e.g. work) an animal will exert to obtain food. Salamone and colleagues examined this using a food choice test, wherein animals were given the option of either lever-pressing to obtain palatable rewards or freely accessing less-desirable chow. At baseline, the reward was highly preferred and animals chose to lever-press for rewards while eating very little chow. However, disruption of DA signaling in the NA shifted their preference to chow, suggesting that loss of NA DA signaling reduced willingness to work for the palatable reward. By contrast, disruption of DA signaling in the dorsal striatum equally reduced both lever-pressing and chow

intake, indicating generalized reduction of feeding regardless of effort[129-131]. Similar results were observed using other behavioral paradigms to parse motivated vs. free intake[132,133], substantiating the hypothesis that NA DA is specifically necessary for "wanting" or the approach phase of motivated behavior. By contrast, DA deficient mice with select restoration of DA to the dorsal but not ventral striatum recover their ability to feed *ad libitum*[134,135], suggesting that dorsal striatal DA is sufficient for food intake that does not require effort. However, this type of free-feeding behavior is not necessarily analogous to "liking". The hedonic responses to food can be assessed in animals through orofacial responses during eating[136], and Berridge argues that "liking" does not require DA at all. Complete destruction of mesolimbic and nigrostriatal DA neurons does not reduce hedonic responses to sweet taste[111]. Furthermore, DA-deficient mice have normal preference for rewards and enhancing NA DA fails to increase "liking" of palatable food, but does increase "wanting"[137,138]. Instead, "liking" may be mediated by mu opioid or GABA-ergic inhibition of anatomically restricted "hedonic hotspots" in the NA shell and ventral pallidum[109].

DA neurons also play important roles in the initial and continued response to cues that may impact feeding. For example, DA neurons are activated in response to unexpected presentation of food, but this response quickly habituates[112,139]. When animals are conditioned to associate a cue with a food reward, DA firing will initially occur during consumption of the reward, however with learning, a shift occurs and DA neurons fire instead during the cue[112], hence DA serves as a signal that predicts delivery of the reward. Rats presented with a novel palatable meal have increased NA DA release during consumption, however when presented with the same meal a second time, DA release is absent during consumption due to habituation. By contrast, if a novel palatable food is presented instead at the second meal, NA DA release increases during consumption, suggesting that DA neurons fire in response to the unexpected palatability of the second meal[140]. Although NA DA release may occur during feeding due to

novelty or unexpected presentation of food, as discussed above, lesion studies have shown that NA DA is not required for free feeding behavior. In line with the idea of reward prediction error, NA DA serves more as a "teaching signal" that codes how valuable a reward is, and therefore, how much work an animal should put forth to attain the reward[113].

While DA release occurs in response to palatable taste transmitted by gustatory pathways [141-143], additional work has demonstrated that taste-independent post-ingestive signals can also trigger mesolimbic DA release and food reinforcement. For example, mice genetically blind to sweet taste still develop preference for sucrose and display DA release in response to sucrose but not saccharin[144]. Given that saccharin is devoid of calories, this suggests that post-ingestive signals, likely released in response to caloric delivery to the gut, are sufficient to induce DA release independently of taste perception. This is supported by recent work showing that striatal DA release also occurs in proportion to increasing concentrations of intra-gastric infusion of lipid emulsions[145], thus taste perception is not necessary for DA release in response to caloric content. In sum, these studies support the view that DA release occurs in response to both 1) sensory perception of taste, especially when novel or more palatable than expected, and 2) delivery of calories to the gut via post-ingestive mechanisms and independently of taste.

Mesolimbic DA neurons and food "wanting" are also modulated with respect to peripheral energy status. For example, food deprivation increases DA neuron excitability, decreases DA re-uptake, and potentiates DA release during re-feeding[62,140,146-149]. Thus energy deficit appears to heighten the sensitivity of the mesolimbic DA system to food, and indeed, food-restricted animals will work harder to obtain food compared to sated controls[150,151]. Interestingly, fasted animals will also work harder to obtain sucrose but not for saccharin, indicating that post-ingestive mechanisms signal the caloric value of ingested rewards to VTA DA neurons[151]. However, after unlimited access to food and subsequent satiety, they diminish

their effort to obtain the palatable rewards[151]. Thus, food "wanting" is modulated by peripheral energy status (e.g. deficiency or satiety) presumably to coordinate food intake needed to ensure survival.

There has been considerable interest in understanding if hormones conveying energy status, such as leptin or ghrelin, directly or indirectly engage VTA DA neurons to modify energy balance. Leptin is a satiety cue secreted from adipose tissue and serves as a long-term signal of peripheral fat stores[40]. Leptin action via the long form of the leptin receptor (LepRb) is also important for suppressing feeding via the hypothalamus, but some part of leptin action may be mediated via the VTA. Indeed, exogenous leptin treatment alters signaling in VTA DA neurons[152,153], but only 6% of VTA DA express LepRb and they project to the amygdala rather than the NA[154]. Furthermore, while peripheral or ICV leptin suppresses food intake, sucrose self-administration, and conditioned place preference for palatable food[150,155-157], the evidence for direct behavioral effects of leptin signaling via the VTA are limited. One study reported that intra-VTA leptin decreased food intake while silencing of VTA LepRb receptors increased it[152], but subsequent work has not supported a direct role for VTA LepRb neurons in regulating feeding[158,159]. Instead, the anorectic effects of leptin may be induced via LHA LepRb neurons that project to the VTA and modulate DA signaling[65,160]. In contrast to leptin, ghrelin is a gastric-derived hormone that increases in the circulation to signify energy deficit and strongly promotes food intake[41]. Ghrelin acts on the growth hormone secretagogue receptor (GHSR), which is also present in the hypothalamus and VTA[161]. Intra-VTA ghrelin increases firing of DA neurons through enhanced excitatory input and increases NA DA release[161-163]. In line with this, ghrelin infusion into the VTA increases both *ad libitum* food intake and self-administration of palatable food[161,164,165] and blocking GHSRs in the VTA or DA signaling in the NA abolishes these effects[161,162,166]. Taken together, there is some evidence for direct modulation of VTA DA

neurons by leptin and ghrelin, but these hormones may also exert control via hypothalamic targets that project to the VTA.

1.3.3 Dopamine and Energy Expenditure

The role of DA signaling in energy balance has been studied extensively in the context of feeding, yet energy expenditure represents an equally important determinant of body weight. It is well-established that pharmacologic enhancement of DA release also induces locomotor activity[167,168] and in fact, psychostimulant-induced locomotor activity is often used as a behavioral surrogate to assess striatal DA signaling. For example, reducing DA re-uptake through pharmacologic or genetic disruption of DAT activity is associated with increased locomotion[169,170]. Similarly, agonist-mediated stimulation of D1R also increases locomotor activity, while D2 agonists increase autoinhibition that tends to suppress locomotor activity[104]. It is interesting that altering mesolimbic DA signaling has a predictable and well-established direct impact on locomotor activity, while the same manipulations rarely alter food intake unless animals are forced to work for food[61,171]. Thus, locomotor activity in general is tightly linked to DA signaling, but certain aspects of food intake may not be. Furthermore, it is difficult to interpret what locomotor activity means in rodents since it varies based on the testing procedure and encompasses aspects of novelty, exploratory, and emotional behaviors[171].

A standard way to examine volitional activity in rodents is to provide access to running wheels. Animals will lever-press for access to wheels and in some cases find it more reinforcing than sucrose[172,173]. Additionally, animals prefer to spend time in environmental contexts that they have been conditioned to associate with running wheels, even when the wheel is not present[174]. These data indicate that volitional wheel running is both rewarding and reinforcing, and therefore may engage mesolimbic DA circuits. Indeed, rodents bred for high levels of physical activity have increased levels of striatal DA and increased expression of D1R[175,176].

Long-term access to wheels also increases TH expression and ΔFosB in the mesolimbic system[174]. Interestingly, food-deprived rats work harder for wheel access and run more when they are on the wheel, suggesting that physiologic state regulates the motivation to run[177]. Recently, Fernandes *et al.* showed that deletion of a critical mediator of leptin signaling specifically in the VTA caused increased volitional activity and preference for running wheels compared to controls[158]. Together, these studies support the hypothesis that the hormonal signature of energy depletion (i.e. lack of leptin signaling or food restriction) may directly act via VTA DA neurons to increase locomotor activity. While it seems counterintuitive that animals would increase energy expenditure in an energy-depleted state, one explanation is that ambulation and exploratory behavior is advantageous in a food-restricted state, as it could increase the probability of finding food. Consistent with this theory, Beeler *et al.* argues that DA is not a feeding signal *per se*, but instead coordinates energy expenditure in terms of how much energy should be allocated towards specific behaviors, including, but not limited to feeding[171]. DA neurons become hypersensitive during food deprivation[146,147,178] in part due to changes in insulin, leptin, and ghrelin levels, thus engaging in reinforcing behaviors such as feeding or running in a fasted state is associated with more DA release and increased effort to attain the reward. This is supported by numerous lines of evidence demonstrating that fasted animals work harder for not only food or running but also for abusive drugs[179-181]. In sum, locomotor behavior is tightly tied to DA release, and may indicate a general increase in attention, arousal, and exploratory behavior that can increase effort for rewards in a context-dependent manner.

1.3.4 Neuropeptide Regulation of Dopamine Signaling

Recent interest has been invested in understanding how neuropeptides modulate midbrain DA neurons, and hence how they direct behaviors relevant to energy balance. For example, pharmacological and electrophysiological studies suggest that VTA DA neurons express receptors for the neuropeptides, orexin, corticotropin-releasing factor, and neurotensin,

which all have the capacity to regulate DA neuronal function and motivated behaviors (reviewed in[182-184]). The specific importance of neuropeptides in directing neuronal signaling is just beginning to be appreciated (as recently reviewed by van den Pol[185]). Neuropeptides are often co-expressed along with the classical amino acid neurotransmitters glutamate or GABA, however the properties of neuropeptides vary greatly from classical transmitters. First, glutamate and GABA are released from the pre-synaptic terminal and travel mere nanometers across the synapse, where they act rapidly on ion channels to activate or inhibit the post-synaptic neuron. By contrast, neuropeptides released form presynaptic terminals can diffuse microns outside the synapse and thus can bind to both the post-synaptic neuron and neighboring neurons that do not make direct synaptic contact. While classical transmitters can gate ligand-gated ion channels to rapidly alter membrane potential, neuropeptides bind to G-protein coupled receptors that change gene transcription or intracellular Ca^{++}, and hence mediate long-term signaling changes in target neurons. Furthermore, in addition to release at the presynaptic terminal, some neuropeptides can be released from the dendrites or anywhere along the axon, which further increases the number of neurons that are influenced by neuropeptide release. Thus, although classical transmitters and neuropeptides may be released from the same neuron, they mediate very distinct and temporally dissociable effects on target cells. Furthermore, the distribution of different neuropeptide receptors on target neurons allows for further refinement of signal transduction and physiologic regulation, and these may be selectively targeted via pharmacological means. Understanding the role of neuropeptide and neuropeptide receptor systems that engage the DA system thus holds promise for selective, long-term control of DA-mediated behaviors that impact body weight.

1.4. Neurotensin Physiology

1.4.1 Discovery, Receptors, and Expression Patterns

Neurotensin (Nts) is a 13 amino-acid neuropeptide that was first isolated from bovine hypothalamus by Carraway and Leeman in 1973. Upon discovery, Nts was first appreciated for its hypotensive effects as the authors noted "visible dilation" when it was applied to blood vessels in anesthetized rats[186,187]. In addition to regulation of blood pressure, Nts has since been implicated in a diverse array of physiologic processes including feeding, locomotor activity, body temperature, and gastric motility[188]. Nts is synthesized as part of a larger pro-peptide that also contains neuromedin-N and is cleaved into biologically active Nts by pro-hormone convertases in the Golgi apparatus and endoplasmic reticulum[189]. In neurons, the processing, storage, and release of Nts has not been well characterized, but similar to other neuropeptides, Nts has been localized in dense core vesicles at pre-synaptic terminals[190].

The discovery of Nts in the brain suggested it exerts central control of physiology, and indeed ICV administration of Nts is sufficient to alter food intake, locomotion, and body temperature[87,191,192]. Nts is also highly expressed within the gastrointestinal tract and adrenal gland, and release of Nts from these tissues presumably accounts for the high levels of Nts in the circulation. However, circulating Nts has an extremely short half-life due to rapid degradation by proteolytic enzymes[193] . Thus, the central effects of Nts are likely due to Nts that is produced by, and released within the brain, but not from peripheral tissues. In the brain, studies using *in situ* hybridization and immunohistochemistry reveal that Nts is highly expressed within the LHA, NA, lateral septum, stria terminalis, preoptic area, central amygdala, periaqueductal grey and parabrachial nucleus[188,194].

Nts binds two G-protein coupled receptors, Nts receptor 1 and Nts receptor 2 (NtsR1 and NtsR2), and Nts has a 10-30 fold higher affinity for NtsR1 than NtsR2[195-198]. NtsR1 is coupled to G_q, which leads to induction of phospholipase C and increased intracellular Ca^{++}[199,200]. The downstream signaling transduction of NtsR2, however, is not well characterized; it may be G_q or G_i-coupled and could vary across species[197,201]. Nts also binds to a third non-G-protein-coupled receptor known as Nts receptor 3 (NtsR3) or sortilin, which consists of a single transmembrane protein but little else is known about its role in Nts signaling[202,203]. *In situ* hybridization and autoradiography reveals that NtsR1 is robustly expressed within the VTA, SN, septum and suprachiasmatic nucleus in adult animals. Interestingly, *Ntsr1* expression is high and extensively distributed throughout the brain during early embryonic stages but is rapidly downregulated within the first few weeks of life, suggesting that Nts signaling via NtsR1 may be involved in the development of neural circuits[204,205]. By contrast, the expression of NtsR2 has been difficult to localize because most *in situ* hybridization studies show low levels of diffuse expression throughout the entire brain, however some have reported notably high levels in the cerebellum, periaqueductal gray, hippocampus, and VTA and SN[206,207]. Furthermore, NtsR2 does not exhibit a transient peak in expression during early development like NtsR1, and instead, is produced at low amounts during gestation and slowly increases to adult levels after birth. Several authors have suggested that NtsR2 is expressed on astrocytes *in vitro*[208-210], however controversy remains as one report found that NtsR2 binding sites do not colocalize *in vivo* with the astrocyte marker, GFAP[211]. Clearly, the differences between NtsR1 and NtsR2 in terms of their affinity for Nts, expression patterns, and ontogeny suggest that they are functionally distinct, and indeed this is supported by work using antagonists or mice with developmental deletion of NtsR1 and NtsR2. In general, NtsR1 is implicated in mediating the feeding, locomotor, reward, and body temperature effects of Nts, while NtsR2 is implicated in Nts-induced analgesia[203,212-218]. The contributions of different Nts

receptors with respect to Nts modulation of DA signaling and energy balance will be discussed in detail in the following sections.

1.4.2 Neurotensin and Dopamine Signaling

Nts regulates DA signaling and has accordingly generated considerable interest as a potential therapeutic target in diseases characterized by disrupted DA action, especially schizophrenia and Parkinson's disease. Generally-speaking, Nts or Nts analogues enhance the activation of DA neurons through three different mechanisms: 1) increased intracellular Ca^{++}[219], 2) decreased D2R-mediated autoinhibition[220-223], and 3) increased presynaptic excitatory input[217,220,224]. While several studies suggest that NtsR1 is required to activate DA neurons, roles for NtsR2 have also been proposed[217,224], hence the precise mechanism by which Nts promotes activation of DA neurons remains unclear.

Pharmacologic studies indicate a specific role for Nts action via the VTA, where it increases NA DA release and locomotor activity similar to psychostimulants[92,214,225-232]. Nts administration to the VTA also supports self-administration[217,233,234], conditioned place preference (CPP)[216,235], and sensitization[236,237], indicating that Nts signaling in the VTA modifies DA-dependent behavior and is rewarding. Loss of NtsR1 signaling abolishes Nts-mediated self-administration and CPP[216,217], suggesting that NtsR1 confers the reinforcing properties of Nts activity in the VTA. In support of this, transgenic *NtsR1*^{Cre} reporter mice indicate that NtsR1 is expressed almost exclusively by DA neurons within the VTA[238]. However, the receptor mechanism by which Nts acts in the VTA remains controversial, since studies using receptor antagonists or receptor-null mouse models have yielded conflicting data on the importance of NtsR1 and/or NtsR2[214,228]. Indeed, the VTA contains mRNA and binding sites for both receptors[206,207,239,240], so both could play roles in modulating DA signaling and behavior. Reagents to permit the visualization of NtsR1 and NtsR2-expressing cells in the VTA would be

useful to determine if the receptor isoforms directly or indirectly modify VTA DA neurons, and hence to understand how Nts exerts control over DA signaling.

While pharmacologic administration of Nts in the VTA strongly influences mesolimbic DA signaling and reward behaviors, the endogenous circuits providing Nts to the VTA, and how they modulate it, remain undetermined. Some Nts-containing neurons must provide Nts to the VTA, as demonstrated by the density of Nts immunoreactive terminals in the midbrain[241-244] that are found in close apposition to VTA DA neurons[238]. Interestingly, Nts itself is present in the VTA and colocalizes with DA markers in rats[244-246], but not in mouse or human[247,248]. Thus in mice and humans, Nts input to the VTA does not originate locally and must therefore come from elsewhere in the brain. Retrograde tracing in rats demonstrated the many Nts afferents to the VTA reside in the preoptic (POA) area, rostral LHA, and NA[182,249], but the functional significance of these Nts inputs remains undefined. Curiously, destruction of the Nts neurons in the POA and LHA, which provided the majority of input to the VTA, did not substantially reduce Nts reactivity in the VTA, suggesting that Nts regulation of DA signaling is highly conserved[250]. Methods to selectively activate Nts neurons that project to the VTA are necessary to reveal how endogenous Nts circuits contribute to behavior. For example, chemogenetic activation of LHA Nts neurons increases NA DA release and locomotor activity[93], similar to the effects of pharmacologic Nts in the VTA. While it will be important to assess the roles of each population of Nts neurons that project to the VTA, these data suggest that LHA Nts neurons mediate at least some aspects of Nts action via the VTA, and deserve further exploration.

1.4.3 Neurotensin and Energy Balance

A first glimpse into the idea that Nts may be involved in energy balance came from work in the early 1980's showing that central administration of Nts suppresses food intake in hungry rats[251]. The central anorectic effects of Nts have since been replicated by multiple independent

groups[87,252-254], and peripheral Nts treatment has also been demonstrated to suppress appetite and reduce body weight [90,252,255,256]. Nts administration directly to the VTA potently suppresses food intake, spurring interest in whether this might be associated with the known roles for Nts in regulating mesolimbic DA signaling[86,88]. Consistent with this, intra-VTA Nts also reduces operant responding for food[88,257] and in most of the studies cited above, animals were either fasted or trained to eat food within a single short time period, both procedures that increase motivation to eat. Nts also suppresses feeding when administered to hypothalamic sites[258], but not the NA[87], indicating that Nts-induced anorexia occurs within specified anatomical circuits. Importantly, repeated peripheral injection of brain-penetrating Nts analogues or NtsR1 agonists suppresses food intake and promotes sustained weight loss in both lean and obese mice[89,90], indicating that the anorectic effects of Nts are sufficient to modify body weight. Nts treatment does not, however, suppress feeding in mice lacking NtsR1[212,253], suggesting that Nts mediates anorectic effects via NtsR1. This finding prompted investigation of whether loss of NtsR1 altered body weight, but independent reports suggest that NtsR1 knockout mice are either overweight[212], not different[213,218], or slightly lighter[238] compared to controls. This variation could be explained by differences in genetic background and environment, similar to how some, but not all individuals with genetic predisposition to obesity become obese. Mice genetically lacking NtsR1 are also more susceptible to weight gain when given high fat diet, implicating NtsR1 signaling in palatable food intake[238].

Interestingly, mounting evidence suggests some overlap in the mechanisms by which the anorectic hormone leptin and central Nts suppress feeding. Leptin treatment increases Nts expression in the hypothalamus[259,260], but disruption of NtsR1 signaling abolishes the ability of leptin to suppress food intake[238,253,261]. Taken together, these data support a hypothesis whereby leptin acts via LepRb-expressing neurons to increase Nts signaling via NtsR1, leading to reduced food intake. Leinninger *et al.* began to investigate this hypothesis by showing that

many LepRb-expressing neurons in the LHA also express Nts, and that the LHA is the *only* place in the brain where LepRb and Nts are co-expressed[65,85]. Deletion of LepRb selectively from LHA Nts neurons leads to increased body fat and impaired coordination of feeding behavior with peripheral energy cues[66,85]. These LHA Nts neurons project to the VTA, where their terminals are in close apposition with DA neurons[85,238]. Furthermore, NtsR1 is expressed almost exclusively on DA neurons in the VTA[238], thus LHA Nts neurons can directly access mesolimbic DA circuits and are poised to regulate DA signaling via NtsR1. Consistent with this, chemogenetic activation of LHA Nts neurons increases DA release to the NA, as well as locomotor activity and energy expenditure that causes weight loss over 12 hours, but no alteration in food intake was observed[93]. This acute study, however, does not rule out a role for LHA Nts neurons in regulating feeding, nor does it indicate whether action via LHA Nts neurons can maintain sustained weight loss. These issues merit further investigation to understand the contribution of LHA Nts neurons to physiology and energy balance.

1.5 Neurotensin and Dopamine Signaling in Energy Balance Disorders

1.5.1 Is Dopamine Disrupted in Obesity?

The hallmarks of obesity include overconsumption of calorically dense food and lack of physical activity, leading to weight gain. Since DA circuits regulate both feeding and volitional activity, many efforts have attempted to establish a causal link between dysfunctional DA signaling and obesity. One explanation for why obese individuals overeat is that they experience less reward in response to palatable foods and overeat to compensate; this is termed the “reward deficiency” hypothesis[262]. An alternative explanation is that obese individuals overeat because they have heightened reward responses to food cues and hence experience more pleasure during ingestion of highly palatable food. Thus, hypersensitive

reward circuits could also drive hyperphagia and weight gain, and this is referred to as the "reward-surfeit" hypothesis[263,264]. Evidence for both of these hypotheses will be discussed below.

Several studies indicate that obese individuals have reduced D2R availability in the striatum[265-268], suggesting rationale for the "reward deficiency hypothesis". Indeed, humans with polymorphisms in the *taq1* gene, which reduces D2R binding by 30-40%, are overrepresented in obese human populations compared to lean controls[269-272]. Furthermore, individuals with these *taq1* variants are more likely to have reduced striatal response to palatable food that is predictive of future weight gain[271,273]. Similar results have been noted in animal obesity models. For example, rodents fed high-fat diets have reduced DA release in the NA, and blunted expression of DA-associated markers[274-277]. Johnson and Kenny showed that rats fed a palatable "cafeteria diet" consisting of "bacon, sausage, cheesecake, pound cake, frosting, and chocolate" have reduced striatal D2R binding and engage in compulsive-like behavior to attain the calorie-dense foods. Experimental depletion of striatal D2R receptors in lean rats mimicked compulsive feeding behavior, but did not alter *ad libitum* cafeteria diet consumption or weight gain, suggesting that lack of D2R signaling may regulate specific facets of feeding behavior but is not sufficient to cause obesity on a calorie-rich diet[278]. Taken together, these data suggest that striatal D2R signaling is blunted in obesity, which could be interpreted as hypoactivity of motivational and reward circuits, lending support to the reward deficiency hypothesis. Despite a seemingly strong link between low D2R and obesity, the idea that reduced D2R signaling is a cause rather than consequence of obesity has been called into question. Conflicting with previous work, several more recent studies have found no link between obese populations and D2R binding[279-283]. Furthermore, whole-body or striatal knockdown of D2Rs decreases locomotor activity but does not predispose animals to weight gain on HF diet[278,284,285], suggesting that reduced D2R signaling may cause lower activity levels in obesity, but is not

sufficient to drive overconsumption on palatable diet. This casts doubt on the reward deficiency hypothesis and suggests that reduced striatal D2R may be a consequence rather than a cause of weight gain.

An alternate explanation is the reward-surfeit hypothesis, which argues that individuals with hyper-responsive reward circuits overconsume food that leads to weight gain and obesity[263,264]. Indeed, obese individuals show a greater response to anticipation and receipt of food in brain areas associated with sensory and hedonic aspects of food[286] and they perceive palatable foods as more pleasant than lean controls[287,288]. Obese individuals also show greater striatal activation in response to visual food cues than normal weight subjects[289,290]. Interestingly, adolescents at risk for obesity show greater striatal activation during receipt of palatable food but not during anticipation, suggesting that initial vulnerability to obesity may explained by hyper-responsive reward circuits during food intake[264]. Thus, via the reward-surfeit hypothesis, increased hedonic and sensory response to palatable foods may initially fuel overconsumption, and repeated intake of palatable foods over time leads to conditioned associations with cues that predict food, which over-activate striatal regions to drive further overconsumption after the onset of obesity[264]. However, none of these neuroimaging studies have implicated DA dysfunction in hyperactive responses to food and food cues in obesity. In fact, increased hedonic response to food is likely mediated via non-DA-ergic mechanisms such as mu opiod receptor signaling or other neurotransmitter systems in both animals and humans[280,281,291]. Although evidence exists for both the reward deficit and reward surfeit hypotheses, like many human diseases, obesity may be a heterogeneous disease that can be caused by either hyperactivity or hypoactivity of the reward system, which could vary with genetics and environment. Consistent with this, humans with genetic reduction in D2R availability had increased risk of future weight gain if they showed *reduced* striatal response to food cues, whereas humans with genetically normal D2R signaling had increased risk of future

weight gain if they showed *increased* striatal response to food cues[273]. Thus, obesity cannot be defined by one mechanism but clearly involves disruption in reward circuitry that encompasses both DA-mediated "wanting" and DA-independent "liking."

A note on food addiction: Many behavioral and biological parallels have been drawn between obesity and substance abuse, leading some to ask whether overconsumption of food in obese individuals is caused by "food addiction." For example, similar to obesity, *taq1* allele polymorphisms are associated with substance abuse disorders and drug addicted subjects show reduced striatal D2R binding[292-295]. Although the neural circuitry disrupted in substance use disorders and obesity may overlap to some degree, Stice *et al.* caution against using the term food addiction[296]. As the authors point out, the substance use disorder often has elements of both dependence (i.e. tolerance and withdrawal) and abuse (use of substance despite negative consequence) and they argue it is difficult to apply these criteria to palatable food. In particular, few studies have shown that animals can develop tolerance and withdrawal symptoms to sugar[297,298]. Furthermore, few people who try addictive substances go on to develop substance use disorder, yet a much higher proportion of individuals who eat palatable foods gain weight and become obese. In the words of Stice *et al.,* "very few humans are driven to violent criminal behavior due to craving for chocolate"[296]. While this may be true, it is important to remember that unlike most addictive drugs, possession and consumption of palatable food is not against the law, thus the immediate negative consequences for obtaining high calorie foods are comparatively minimal. In fact, consumption of calorically-rich food is socially acceptable and often *expected* (i.e. cake and ice cream at birthday parties), plus food intake in general is necessary for survival. Thus, it is difficult to directly compare food to addictive drugs as "substances" that have abusive potential. In sum, although obesity and substance abuse may share some common features, care should be taken in considering

whether obese individuals are “food addicts” especially in the context of how medical professionals define addiction.

1.5.2 Evidence for Neurotensin in Obesity

As described previously, Nts suppresses food intake when administered peripherally or centrally, including directly to the VTA, and this is likely mediated by NtsR1. Nts also increases locomotor activity through NtsR1 when delivered to the VTA, but not centrally or peripherally[92,191,299]. Chronic Nts or NtsR1 agonists may therefore have therapeutic potential to induce weight loss if delivered to the VTA, but this has not been examined. The physiological role of Nts in energy balance also remains unclear. To date, a handful of studies have shown reduced levels of Nts protein or mRNA in the hypothalamus of genetically obese mice and rats[300-303]. Given the anorectic effects of pharmacologic Nts, reduced levels in obesity might lead to increased food intake, but there is insufficient data to evaluate whether decreased hypothalamic Nts content is a cause or effect of long-term hyperphagia. Peripheral injection of Nts analogs or NtsR1 agonists are sufficient to suppress food intake and either induce weight loss or prevent weight gain in lean rodents[89,90]. Interestingly, these compounds also restrain feeding and reduce body weight in genetically obese rodents, indicating that Nts may have translational potential as an anti-obesity agent[89,90]. In both of these studies however, the obese animal models were genetically deficient in leptin signaling; given the overlap between leptin and Nts action, inducing Nts signaling might have simply rescued their disrupted leptin-induced Nts signaling to potentiate weight loss. The anorectic potential of Nts in diet-induced obesity, which is the common cause of human obesity, has yet to be examined. Peripheral Nts or intra-VTA Nts suppresses operant responding for sucrose[257,304], suggesting that Nts may indeed be capable of restraining intake of palatable, obesogenic foods. Furthermore, mice genetically lacking NtsR1display increased sucrose preference and susceptibility to weight gain on palatable diet but not chow, implicating the requirement of NtsR1 for restraint of hedonic

intake[238]. Collectively these data suggest potential for Nts signaling as a novel target system for weight control, but much additional work is necessary to determine the precise circuits by which Nts acts to modify feeding and energy expenditure.

In 2016, Nts received considerable attention in the context of obesity when Evers *et al*. published in *Nature* that Nts knockout mice were protected from obesity on high fat diet. This report focused on peripheral Nts, suggesting that Nts produced within the gastrointestinal tract is essential to facilitate intestinal fat absorption. As a result, loss of Nts-mediated fat absorption led to protection from diet-induced obesity. The authors also showed that circulating Nts levels were increased in the blood of obese individuals, and that elevated Nts in lean individuals predicted future weight gain[305]. While this study implicates an important role for peripheral Nts in energy accumulation, it also underscores the importance for site-specific investigation of Nts action to discern its contributions to physiology. The potentially differing roles of Nts in the brain (to suppress feeding) compared to the gut (to promote fat absorption) must also be taken into account in the development of future pharmaceuticals that target the Nts system. Given the very short half-life of circulating Nts, and the unlikelihood of the neuropeptide to traverse the blood brain barrier, the central effects of Nts are unlikely to be mediated by peripherally-generated Nts. As such, Nts signaling in the gut is outside the scope of this dissertation, which instead focuses on elucidating the mechanisms by which central Nts engages the DA system to modify behavior and body weight.

1.6 Goals of the Dissertation

Pharmacologic Nts modifies VTA DA signaling, increases physical activity, and restrains food intake, but the endogenous circuits by which Nts mediates these effects to regulate energy balance remain unclear. Previously, understanding of the physiological roles of Nts in engaging DA signaling was limited by the lack of reagents to visualize and manipulate specific populations

of Nts-expressing and Nts-regulated cells. To overcome this obstacle, we used existing mice that express Cre-recombinase in Nts neurons, and we developed mice that express Cre in NtsR1 or NtsR2 cells; these models allow us to reveal and interrogate specific Nts circuits using Cre-inducible molecular tools. Better understanding of the precise mechanisms by which Nts acts to coordinate food intake and energy expenditure will lead to novel therapeutic targets for the prevention and treatment of obesity. As such, the major goals of this dissertation are:

1. Define the Endogenous Nts Inputs to the VTA (Chapter 2)

Hypothesis: The VTA receives afferent input from select anatomically-defined Nts populations that regulate reward rather than aversion.

Method: We utilized retrograde tracing in mice expressing GFP selectively in Nts neurons to identify all Nts neurons that project to the VTA, and hence revealed which neural populations are anatomically positioned to mediate the weight-reducing effects of Nts within the VTA.

2. Decipher the Neurotransmitter Content, Ontogeny, and Projections of VTA NtsR1 and NtsR2 Neurons (Chapter 3)

Hypothesis: NtsR1 is the dominant neuronal isoform by which Nts regulates DA signaling.

Method: We utilized a dual recombinase strategy to reveal the VTA cells that express NtsR1 and NtsR2 during development and in the adult brain, and their projection sites. This study reveals that NtsR1 and NtsR2 are expressed by different VTA cell types, and implicate NtsR1 as the predominant receptor isoform on VTA DA neurons.

3. Understand How Loss of Nts Input to the VTA Modifies Energy Balance (Chapter 4)

Hypothesis: NtsR1 signaling in the VTA is critical for coordination of behaviors that impact body weight.

Method: Genetic ablation of NtsR1 neurons in the VTA revealed the requirement for Nts signaling via the DA-ergic VTA NtsR1 neurons, and hence for control of DA-mediated behaviors that coordinate body weight.

4. Determine How LHA Nts Neurons Regulate Feeding and Body Weight (Chapter 5)

Hypothesis: LHA Nts neurons regulate energy balance by engaging the mesolimbic DA system via NtsR1.

Method: DREADD technology was used to selectively activate LHA Nts neurons in mice that have intact and disrupted NtsR1, permitting examination of how LHA Nts→NtsR1 signaling contributes to feeding behavior and energy expenditure in lean and obese mice.

Through these goals (Fig. 1), we establish a central circuit whereby LHA Nts neurons act via VTA NtsR1 neurons to coordinate feeding and volitional activity that modify body weight. These data establish a novel, physiologic mechanism to explain the anorectic and locomotor effects induced by pharmacologic Nts, and the translational implications of these findings for treating overweight and obesity will be discussed in **Chapter 6.**

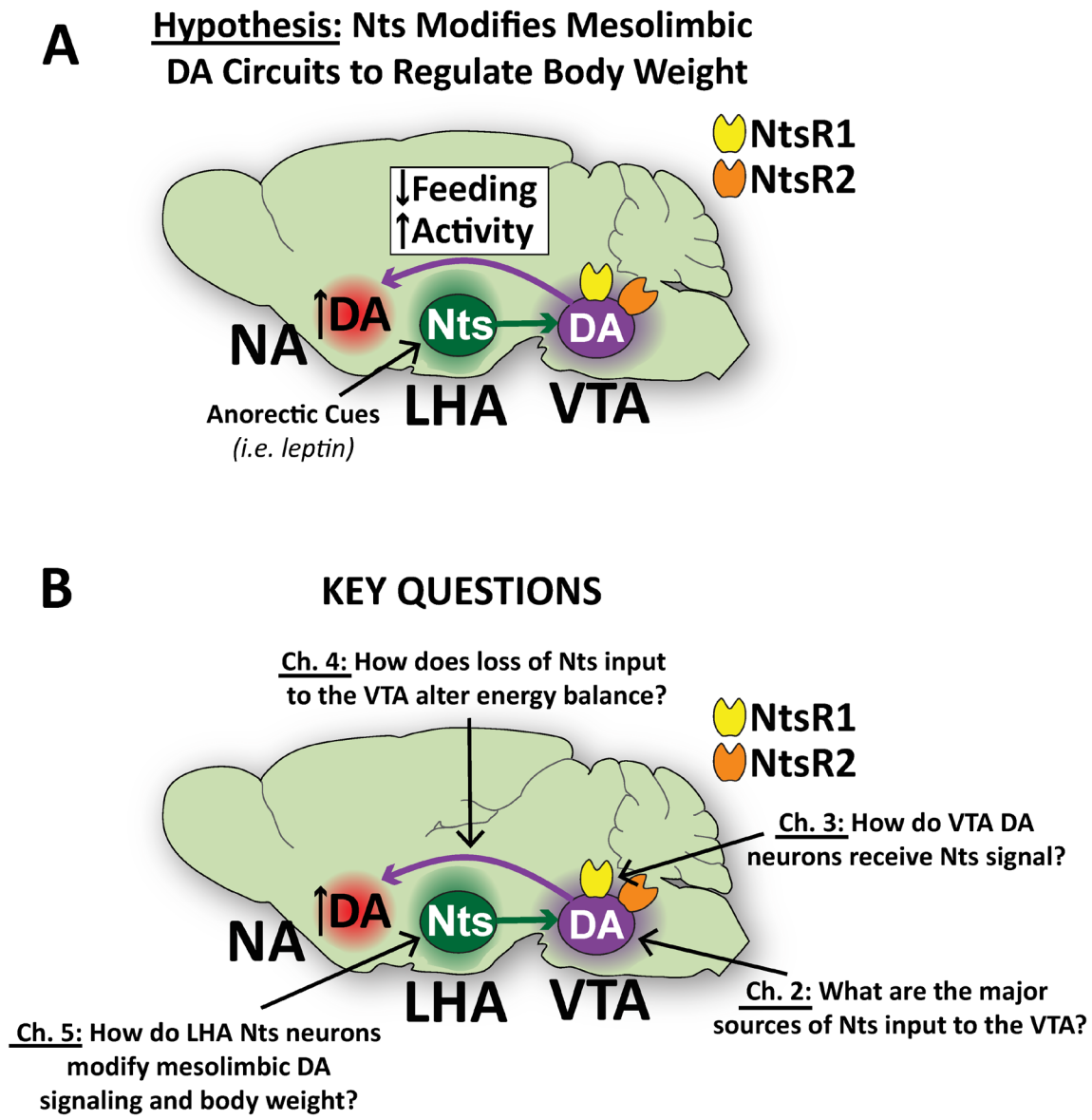

Figure 1. Schematic of hypothesized neural circuit by which Nts acts to modify mesolimbic DA signaling and body weight. A) Hypothesis for dissertation: Nts regulates mesolimbic DA signaling via Nts receptors, and input from the LHA is critical for anorectic effects of Nts. **B)** Key questions addressed in each chapter of the dissertation to investigate how Nts regulates DA signaling energy balance.

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Chapter 2. Determination of Neurotensin Projections to the Ventral Tegmental Area in Mice

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This chapter is a modified version of a manuscript under review.

2.1 Abstract

Pharmacologic treatment with the neuropeptide neurotensin (Nts) modifies motivated behaviors such as feeding, locomotor activity, and reproduction. Dopamine (DA) neurons of the ventral tegmental area (VTA) control these behaviors, and Nts directly modulates the activity of DA neurons via Nts receptor-1. However, the endogenous sources of Nts to the VTA remain incompletely understood, impeding determination of which Nts circuits orchestrate specific behaviors. To overcome this obstacle we injected the retrograde tracer fluorogold into the VTA of mice that express GFP in Nts neurons. Identification of GFP-Nts cells that accumulate fluorogold revealed the Nts afferents to the VTA in mice. Similar to rats, most Nts afferents to the VTA of mice arise from the medial and lateral preoptic areas (POA) and the lateral hypothalamic area (LHA), brain regions that are critical for coordination of feeding and reproduction. Additionally, the VTA receives dense input from Nts neurons in the nucleus accumbens shell (NAsh) of mice, and minor Nts projections from the amygdala and periaqueductal gray area. Collectively, our data reveal multiple populations of Nts neurons that provide direct afferents to the VTA and which may regulate specific aspects of motivated behavior. This work lays the foundation for understanding endogenous Nts actions in the VTA,

and how circuit-specific Nts modulation may be useful to correct motivational and affective deficits in neuropsychiatric disease.

2.2 Introduction

Neurotensin (Nts) is a 13 amino-acid neuropeptide that was first extracted from the bovine hypothalamus by Carraway and Leeman. Nts has subsequently been identified throughout the brain[1-4] and has been implicated in regulating a diverse repertoire of physiology and motivated behaviors including feeding, locomotor activity, social behavior, sleep, and response to addictive drugs[5-10]. Nts may direct certain behaviors via actions in the ventral tegmental area (VTA), based on findings that different types of VTA neurons orchestrate distinct goal-directed behaviors[11-13]. The VTA is predominantly comprised of dopamine (DA) neurons that project to and release DA in the nucleus accumbens (NA), prefrontal cortex (PFC), hippocampus, or amygdala to modify goal-directed behaviors (see[14,15] for review). The VTA also contains GABA-ergic neurons including those within the newly defined "tail" of the VTA or rostromedial tegmental nucleus (RMTg) that locally inhibit VTA DA neurons, thus serving as negative regulators of DA-mediated behaviors[12,16-18]. Given that alterations in both VTA DA and Nts signaling have been implicated in the pathogenesis of drug addiction, depression, anxiety, schizophrenia, autism, and obesity[6,8,19-28], there is likely some functional overlap of the DA and Nts signaling systems. It is therefore critical to define the precise neural mechanisms by which Nts engages the VTA to understand how it regulates diverse motivated behaviors, and how they become maladaptive in disease.

Numerous lines of evidence support a role for Nts acting directly within the VTA to modify behavior. Nts signals via the G-protein coupled receptors, Nts receptor 1 and 2 (NtsR1 and NtsR2), both of which are expressed within the VTA of rodents and humans[29-38], including on some VTA DA neurons[31,38-41]. Pharmacologic administration of Nts or Nts analogs into the

VTA activates DA neurons[37,42-46] and increases DA release in the NA[43,44,47,48]. Mechanistically, Nts may promote DA signalling via direct stimulation of NtsRs on DA neurons, by enhancing excitatory input to VTA DA neurons and/or by decreasing D2 receptor-mediated autoinhibition[42,46,49-51], all of which can lead to DA release that modifies behavior. Indeed, Nts within the VTA suppresses homeostatic and motivated feeding[52,53] and increases locomotor activity[47,48,52,54-58], identifying dual VTA-mediated behaviors by which Nts modifies energy balance. Pharmacologic Nts in the VTA also supports self-administration[50,59,60], conditioned place preference (CPP) [61,62] and locomotor sensitization similar to addictive drugs[47,56,63,64], suggesting that Nts may be rewarding. Furthermore, blockade of Nts receptor signalling disrupts or delays sensitization to amphetamine, cocaine, and morphine[58,65-67]. Intriguingly, many of these behavioral effects are specific to the VTA because Nts administration outside of the VTA elicits different effects. For example, while Nts injection in the VTA increases locomotor activity and DA release, intra-NA or central Nts decreases locomotor activity and does not alter DA release[48,68-72]. Similarly, intra-VTA Nts does not alter acute locomotor response to psychostimulants[56], whereas ICV or intra-NA Nts reduces psychostimulant-induced hyperactivity[73-76]. One notable exception to this is Nts-mediated suppression of feeding behavior, as both central and direct administration of Nts in the VTA suppress feeding in both fasted and satiated animals[52,77,78]. Taken together, these data indicate that Nts can act directly in the VTA to regulate motivated locomotor and reward behaviors.

Although it is clear that exogenous Nts impacts the mesolimbic DA system, the endogenous sources of Nts input to the VTA remain incompletely understood. Abundant Nts-immunoreactive terminals are found within the VTA[38,40,79,80], including in close apposition to VTA DA neurons[39], indicating that some Nts is released to the VTA. Nts afferents to the VTA were examined in rats by injecting the retrograde tracer fluorogold (FG) into the VTA and using

in situ hybridization (ISH) to label Nts cell bodies, demonstrating that most Nts-ergic inputs to the VTA originate from the preoptic area (POA) and rostral lateral hypothalamic area (LHA)[81]. Yet, lesioning the POA and LHA in rats does not substantially reduce Nts terminals in the VTA, suggesting there may be other important sources of endogenous input[82]. Indeed, injection of the wheat germ agglutinin transynaptic tracer into the VTA of rats confirmed afferents from the POA and LHA, and identified putative afferents from the NA shell, dorsal raphe (DR), ventral endopiriform area, lateral septum (LS), pedunculopontine tegmental nucleus (PPTg), and laterodorsal tegmental nucleus (LDTg)[83]. Wheat germ agglutinin can move both retrogradely and anterogradely, however, so some of these sites may be targets of VTA neurons rather than providing afferents[84,85]. Further studies were limited by the inability to easily identify Nts-expressing cells, but the recent development of *Nts*^{Cre} mice enables the facile detection and manipulation of Nts neurons using cre-lox technology. Using these mice we have identified a large population of Nts neurons in the LHA that project to the VTA, consistent with the prior afferent mapping done in rats, and manipulation of these LHA Nts neurons reveals their crucial contributions to motivated behavior and energy balance[39,86-88]. The LHA may be just one of several sites by which Nts orchestrates distinct behavioral responses in the VTA, thus it will be important to define all Nts afferents to the VTA and test their roles individually. Since mice and rats differ in Nts expression[89], they may also differ in the distribution of Nts afferents to the VTA, thus it is crucial to characterize these in mice. We therefore defined the Nts neurons that project to the VTA in mice by injecting the retrograde tracer FG into the VTA of *Nts*^{Cre}*;GFP* reporter mice, permitting robust, simultaneous detection of Nts neurons and VTA afferents.

2.3 Results

The VTA Contains a Small Population of Non-Dopaminergic Nts Neurons: Nts neurons have been previously difficult to identify because immunohistochemical reagents label fibers,

but are insufficient to label cell bodies unless animals are pre-treated with the axonal transport inhibitor, colchicine[88]. To overcome this, we bred mice expressing Cre-recombinase in Nts neurons[88] to a Cre-inducible L10-eGFP reporter[90], producing progeny that express GFP selectively in Nts neurons (we refer to these as *Nts^{Cre};GFP* mice). To understand whether the VTA itself could serve as a local source of Nts input, we first examined the VTA of *Nts^{Cre};GFP* mice for GFP expression. We observed a small population of Nts neurons within the boundaries of the VTA (372 ± 44 total neurons), which were predominantly localized in the caudolateral region (Fig. 2). Interestingly, 97% of Nts neurons within the VTA did not colocalize with TH, indicating that most VTA Nts neurons are not DA-ergic. Since VTA neurons that express GABA or glutamate can project to VTA DA neurons[91,92], these data support the possibility that VTA Nts neurons could locally regulate neighboring VTA neurons. Given the relatively small number of VTA Nts neurons, but the substantial behavioral modifications induced by pharmacological Nts administration to the VTA, we hypothesized that other Nts populations may provide more substantial Nts input to the VTA.

Characterization of Nts Neurons that Project to the VTA: To identify all Nts neurons that project to the VTA, we stereotaxically injected *Nts^{Cre};GFP* mice with the retrograde tracer FG, which labels VTA afferents throughout the brain. Any cell body found to contain both FG (magenta) and GFP (green) represents a Nts neuron that projects to the VTA (gray) (Fig. 3A). We verified VTA targeting by mapping the locations of the midbrain injection tracts and by carefully assessing FG labeling within brain regions known to preferentially project to the VTA vs. the SN, based on the previous work of Watabe-Uchida *et al*[93]. For example, at the level of the hypothalamus, VTA afferents are found in the LHA and zona incerta (ZI), but not in the neighboring dorsal striatum (DS), while SN afferents reside preferentially in the DS. Similarly, the NA heavily innervates the VTA, while the DS preferentially innervates the SN[93] (Fig. 3B). Out of 10 animals injected with FG, 5 had injection sites within the VTA and had many FG-

labeled neurons in the LHA, ZI, and NA, but not the DS (Fig. 3C-E); these 5 mice were subsequently analyzed to define Nts afferents to the VTA. Although the injection tracts of cases F19 and F29 appeared on the lateral border of the VTA, these mice were included for analysis because their FG expression was consistent with VTA rather than SN targeting (e.g. robust in the LHA, ZI and NA, with minimal FG+ neurons in the DS). While our analysis revealed substantial numbers of GFP-labeled Nts neurons and FG-labeled neurons throughout the brain, we will describe only the select brain regions containing colocalized neurons, henceforth referred to as Nts/FG+ neurons.

Striatum and Septum: We observed many Nts/FG+ neurons in the NA shell (NAsh) between +1.75 and 0.85 bregma, but few Nts/FG+ in the NA core (Fig. 4A and Fig. 10). We investigated whether these Nts/FG+ neurons localized to a specific NAsh subregion that might suggest their function, given that site-specific cells along the rostrocaudal NAsh mediate behaviors with either positive or negative valence[94]. The Nts/FG+ neurons, however, were found within a continuous band across the rostrocaudal axis of the NAsh, with no clear localization to rostral or caudal regions. While Geisler and Zahm reported a minor NAsh Nts input to the VTA in rats[83], our quantitative analysis in mice identifies the NAsh as one of the most significant contributors of Nts input to the VTA (Fig. 10). GFP-labeled neurons were also abundant in the olfactory tubercle (OFT), a structure considered to be ventral extension of the NA with similar roles in DA-mediated motivated behaviors[95]. However, the OFT does not provide significant VTA afferents in mice[93,96] and accordingly, we detected few FG-labeled neurons in the OFT (Fig. 4B), suggesting that OFT Nts neurons project to and act at sites other than the VTA.

Compared to the ventral striatum, the dorsal striatum contained relatively few Nts neurons, which clustered around the lateral and medial borders of the caudate/putamen (CPu)

(Fig. 4C, D). The CPu preferentially projects to the SN with minimal afferents to the VTA[93,96]; similarly, we observed some FG+ neurons in the ventral CPu between +1.94 and 0.62 bregma, but the vast majority did not colocalize with GFP (Fig. 4C). Nts neurons in lateral septum (LS) project to the VTA in rats[83], but we observed ≤1 Nts/FG+ neuron per coronal section in the LS of mice. While Nts neurons were abundant in the LS, there were few FG+ neurons (Fig. 4D), consistent with previous work that the LS does not send substantial projections to the VTA in mice[93,96].

Cortex and Amygdala: The neocortex contained relatively few GFP-labeled Nts neurons with the exception of a prominent band of Nts neurons in the prefrontal cortex (PFC). The PFC is the predominant source of cortical input to the VTA, while the lateral orbital cortex (LO) also provides afferents specifically to VTA DA neurons[93]. We noted distinct populations of FG-labeled or Nts neurons in separate layers of the PFC and the LO, but no Nts/FG+ neurons (Fig. 5A, B). Thus, neither the PFC nor the associated neocortex are significant sources of Nts input to the VTA, indicating that they mediate control of the VTA via other, non-Nts signals.

Examination of the amygdala revealed FG-labeled neurons in the central amygdala (CeA) and extended amygdala (EA) with relatively few in the basolateral amygdala (BLA), consistent with previous reports[93,96,97]. Similar to findings from rats, we detected a concentrated area of Nts neurons in the CeA of mice, which were more numerous in the lateral division in caudal sections (Fig. 5C). Some CeA Nts neurons project to the VTA, and were predominantly found between -1.30 and -0.80 bregma (Fig. 5D). A smaller population of Nts/FG+ neurons was observed in the EA. Together these data indicate that the amygdala provides modest Nts input to the VTA in mice (Fig. 10).

IPAC, Pallidum and Stria Terminalis: The interstitial nucleus of the posterior limb of the anterior commissure (IPAC) is closely related to both the ventral striatum and the amygdala[98,99] and also provides afferents to the VTA[93,96]. We noted a few Nts/FG+ neurons in the lateral IPAC (Fig. 6A), which appeared to represent a caudal extension of the Nts/FG+ neurons described in the NAsh. By contrast, Nts expression was consistently scarce in the ventral pallidum (VP) and globus pallidus (GP) (Fig. 6A, B), coinciding with previous work showing low levels of Nts in these structures[3,100]. A few Nts/FG+ neurons were found in the VP, especially near the border with the LPO (Fig. 10). In rats, the stria terminalis (ST) provides Nts afferents to the VTA[83], and similarly we identified a few Nts/FG+ neurons in the ST of mice. Together, the ST, VP, and IPAC provided relatively minor Nts inputs to the VTA (Fig. 10).

Hypothalamus: Compared to other brain regions, the hypothalamus was the largest contributor of Nts neurons that project to the VTA, with most Nts/FG+ neurons located in the preoptic area (POA), LHA, or zona incerta (ZI). The LHA is a key site for integration of peripheral energy cues with motivated behaviors (see [5] for review) and contained a robust population of Nts neurons between -1.30 to -1.70 bregma. Many of these LHA Nts neurons were labeled with FG from the VTA (Fig. 7B), consistent with other reports that LHA Nts neurons directly project to the VTA[39,87,101], and supporting a mechanism by which LHA Nts neurons can coordinate VTA-mediated behaviors such as feeding. Compared to the LHA, other hypothalamic sub-regions contained sparser populations of Nts neurons, including the dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), and paraventricular hypothalamus (PVH) (Fig. 7B-D). Similar to rats[83], we identified a few Nts/FG+ neurons in the ARC and PVH of mice, though the number of ARC and PVH Nts afferents to the VTA are minor compared to the robust number of Nts afferents from the LHA (Fig. 10). The ZI runs along the dorsal border of the LHA but appears to be functionally unique from the LHA and neighboring thalamic areas[102]. Interestingly, while there are minimal Nts

neurons in the ZI, many of them are Nts/FG+ (Fig. 7A, top panel), such that the ZI and LHA contain comparable numbers of Nts neurons projecting to the VTA (Fig. 10). Our examination also revealed a dense population of Nts neurons in the neighboring subthalamic nucleus (STN) (Fig. 7A, bottom panel), but we did not observe FG+ neurons in the STN, which is more likely to provide afferent input to the SN than the VTA[93]. Indeed, the absence of FG labeling in the STN further verified that FG injections were confined to the VTA in the mice analyzed for this study.

Along with the LHA and ZI, the POA provided a large number of Nts afferents to the VTA, whose distribution varied along the rostro-caudal axis. In the caudal POA, the Nts population was most dense in the medial preoptic area (MPO) adjacent to the third ventricle. Many Nts/FG+ neurons were found in the MPO (Fig. 7E) and these VTA-projecting neurons formed a dense cluster centered between -0.22 and +0.02 bregma. Our finding of POA Nts afferents to the VTA in mice is consistent with the recent description of MPO Nts neurons that regulate social reward via the VTA[103]. Collectively, this data and our work characterizing LHA Nts neurons that project to the VTA[39,87,88] provides support for the idea that region-specific Nts input to the VTA controls distinct motivated behaviors (e.g. POA Nts modifies social interaction while LHA Nts modifies energy balance). In the rostral POA the Nts population was sparse in the MPO but dense within the lateral preoptic area (LPO), where we observed many Nts/FG+ neurons (Fig. 7F) spanning between -0.10 and +0.40 bregma. One potential role for these LPO Nts neurons may be initiation of locomotor activity, as LPO-mediated locomotor activity is blunted by microinjection of Nts receptor antagonists in the VTA[104].

Midbrain and Hindbrain: Brainstem nuclei provide direct sensory afferents to the VTA, thus our identification of Nts/FG+ neurons throughout the midbrain and hindbrain suggests roles for Nts in conveying sensory information to the VTA. First, examination of the dorsal midbrain revealed Nts/FG+ neurons in the superior colliculus (SC) and anterior prectectal nucleus (APT)

(Fig. 8A, B), midbrain regions involved in visual and somatosensory processing, respectively[105,106]. Dense populations of GFP-labeled Nts neurons were noted in the parabrachial nucleus (PBN) and the DR, but few of these contained FG, and thus provided minimal projections to the VTA (Fig. 8F, G). The lateral PBN contained segregated groups of either GFP or FG-labeled neurons with the GFP-labeled neurons clustered more laterally, and most co-labeled neurons were found in the middle where the two populations overlap (Fig. 8G). Nts neurons in the DR were most abundant in caudal sections between -4.8 to -5.0 bregma, which also gave rise to the highest numbers of colocalized neurons per section. VTA afferents arising from the pedunculopontine nucleus (PPTg) and laterodorsal tegmentum (LDTg) are associated with reward behaviors[11,107] and we detected small numbers of Nts/FG+ neurons in these regions (Fig. 8D,H). The periaqueductal grey (PAG) contained the highest number of brainstem Nts afferents to the VTA, which were evenly distributed throughout its rostrocaudal extent. Thus while only 4-6 Nts/FG+ neurons were present in each coronal section of the PAG, the cumulative neurons observed across the large rostrocaudal span of the PAG (approximately 2.5 mm) amounted to an appreciable number of Nts afferents from this structure.

Habenula and RMTg: Both the lateral habenula (LHb) and RMTg provide afferent input to the VTA that inhibits DA neurons and mediates aversive behaviors[11,108]. Although most literature strongly suggests that Nts action in the VTA supports reward rather than aversion[50,61,62], we examined the LHb and RMTg to assess whether Nts is co-expressed in aversive inputs to the VTA. Analysis of the LHb revealed many FG+ neurons, but sparse GFP-Nts neurons, and few LHb neurons contained both labels (Fig. 9A). When quantified, we found the LHb Nts neurons provided minimal afferents to the VTA, similar to the modest input from the DMH, VMH and AHA (Fig. 10).

The anatomic boundaries of the RMTg in mouse were recently mapped onto the Allen Brain Atlas[109], and we used these to locate the RMTg in our experiments. In the caudal RMTg, FG+ neurons occasionally colocalized with GFP, but the majority were GFP-negative (Fig. 9B), suggesting that Nts neurons only occasionally provide aversive input to the VTA. Assessment of rostral RMTg Nts afferents was hindered by its close proximity to the FG injection site, and due to the mechanical tissue damage in this region we could not determine whether RMTg Nts neurons also contained FG. Examination of the rostral RMTg in uninjected *Nts^{Cre};GFP* reporter mice, however, revealed an average of 3-4 TH-negative Nts neurons per coronal section (Fig. 9C, D). Thus although we cannot determine whether these RMTg Nts neurons project locally, their modest number suggests that Nts is unlikely to mediate aversion via input to the VTA.

2.4 Discussion

Here, we characterized the endogenous sources of Nts that can directly regulate the VTA in mouse. Our findings reveal that the largest source of Nts input to the VTA comes from the hypothalamus, predominantly from the LPO, MPO, and LHA. The NAsh also provides significant Nts afferents to the VTA, while the amygdala, ST, VP, and sub-regions of the hindbrain provide more modest Nts input. To our knowledge, this is the first comprehensive definition of mouse Nts afferents to the VTA, and is a first step toward understanding how Nts coordinates a diverse repertoire of behaviors. Indeed, since Nts afferents from the MPO and LHA modify DA-mediated social interaction and energy balance, respectively, it is possible that each Nts circuit orchestrates a specific motivated behavior via the VTA. Going forward it will be important to determine the unique roles of each Nts projection, and hence precise mechanisms to influence feeding, locomotor activity, social behavior, sleep, and response to addictive drugs.

While the VTA receives input from many brain structures[93,96,110,111], the Nts afferents were confined to a limited number of regions. For example, the DR, VP, and PBN provide major

input to the VTA [96,112], and we also observed many FG-labeled neurons in these regions but very few were Nts-specific VTA afferents. By contrast, the highest number of Nts-labeled VTA afferents came from the POA (the LPO and MPO, specifically), yet the POA only provides ~2% of all VTA input in mouse[96]. Overall, our data support Geisler and Zahm's hypothesis that Nts inputs to the VTA preferentially originate from regions associated with reward rather than aversion[83]. Indeed, in mice we find that the largest Nts projections to the VTA originate from the POA, LHA and NAsh, regions that are essential for mediating ingestive and social reward behavior[113-115]. By contrast, we detected few Nts afferents from aversion-inducing areas that project to the VTA, such as the LHb and RMTg[11,116,117]. In sum, these data identify specific neural circuits by which Nts may be released to the VTA to mediate positive reinforcement[50,59,61,62].

Comparison to Previous Work: Our work in mice, taken together with tracing studies in rats, confirms that the LHA and POA are the predominant endogenous sources of Nts to the VTA of rodents[83]. We did, however, note several discrepancies in the distribution of Nts afferents in mice compared to those in rats, indicative of species differences in Nts signaling. For example, rats have a continuous band of Nts afferents from the LPO and rostral LHA, while mice exhibit significant clusters of Nts afferents in the perifornical LHA (between -1.30 and -1.80 from bregma) and the LPO with sparse afferents between these regions. Additionally, although mice and rats have many Nts neurons in the LS and endopiriform areas[118,119], mice lack the substantial Nts-afferents to the VTA observed in rats. This discrepancy may be explained by the general paucity of VTA projections from the LS and endopiriform areas in mice (0.1-0.3% of all VTA afferents[96]) compared to rats (~2-3% of all VTA afferents[112]). Thus, Nts from the LS and endopiriform regions of mice and rats likely act via distinct targets, and may differentially influence physiology. Additionally, while the DR provides significant Nts input to the VTA in rats, mice have only modest Nts projections from the DR and other mid- and hindbrain nuclei (e.g.

the LDTg, PBN, PPTg). We did, however, detect small populations of VTA-projecting Nts neurons in the APTN, PAG, and SC, which were not previously identified in rats. Additionally, our mouse data are consistent with reports of rat Nts afferents to the VTA from the NAsh, ST and amygdala, though the density of afferents differs between species. Overall, our data reveal species-conserved Nts circuits from the LHA and POA that can engage the VTA, but also point to other species-specific mechanisms for Nts action that may have functional importance.

We also addressed whether there are Nts neurons within the VTA that provide a local source of Nts. Previously, immunohistochemistry was used to identify a large population of Nts cell bodies that co-express TH, and hence are DA-ergic[79,120] but very few Nts-expressing cells are detected in the VTA via ISH[100]. This discrepancy may be explained by the subsequent recognition that immunoreagents do not reliably detect Nts in cell bodies without the use of colchicine treatment to inhibit axonal transport. Using *Nts^{Cre};GFP* reporter mice, we observed only a small population of GFP-labeled Nts neurons within the VTA, similar to *Nts* ISH. Only 3% of these VTA Nts neurons co-expressed TH, thus the vast majority are not DA-ergic. VTA Nts neurons may instead contain either glutamate or GABA and project locally within the VTA to directly regulate DA neuron firing[91]. Intriguingly, the small number of TH-negative Nts neurons was primarily detected in the caudal VTA along the rostral lateral borders, where the majority of VTA GABA neurons reside[121]. These data suggest that VTA Nts neurons may be GABAergic, and could provide local inhibitory control over neighboring DA neurons. Overall, our finding of few local VTA Nts neurons compared to the robust Nts input from the POA, LHA and NAsh suggests that non-VT sources of Nts are the predominant mechanism to modulate the VTA and motivated behaviors.

Functional Implications of Nts Afferents to the VTA from the POA: Our results reveal that the both the MPO and LPO subregions of the POA contribute substantial Nts input to the

VTA. The POA is sexually dimorphic[122,123], and in female rodents MPO-mediated regulation of the mesolimbic DA system influences mating and care of offspring[124-126]. Nts signaling from the MPO may play a role in maternal behaviors, given that Nts expression in the MPO is altered post-partum[127] and in maternal aggression[128], and mouse MPO Nts neurons are activated during interaction with pups[129]. Nts in the POA may also mediate mating behavior. Indeed, Nts immunoreactivity in the VTA is positively correlated with sexually-motivated behaviors in European starlings[9,130], while optogenetic stimulation of MPO Nts neurons projecting to the VTA mediates social attraction and mesolimbic DA release[103]. Taken together, these data suggest that the dense MPO Nts circuit to the VTA is a mechanism by which Nts can modify mating, parenting, and other social behaviors.

The LPO provides similarly large numbers of Nts afferents to the VTA as the MPO, yet much less is known about the role of the LPO coordinating motivated behaviors. The LPO is implicated in fluid balance and thirst regulation[131], thus it is possible that the abundant Nts neurons in the LPO may modify water intake. However, while central administration of Nts increases water intake[132-134], application of Nts directly into the VTA does not alter drinking behavior[52], suggesting that LPO Nts neurons may modify drinking via other non-VTA targets. The LPO has also been implicated in regulating locomotor activity. Dis-inhibition of the LPO and rostral LHA continuum in rats induces locomotor activity, which is prevented by blockade of NtsR1-signalling in the VTA[104]. These data support a role for endogenous LPO Nts release to the VTA in mediating locomotor activity, and may account for the induction of locomotor activity observed after pharmacologic administration of Nts into the VTA. Given the role of the LPO in drinking and locomotor behavior, it will be important to determine whether the large number of LPO Nts afferents to the VTA selectively modulate these, or other motivated behaviors.

Nts released from the POA may also modify sleeping behavior via the VTA. Modulation of VTA DA neurons directly regulates arousal, such that the activation of VTA DA neurons promotes wakefulness while inhibition drives sleep and sleep-oriented nesting behaviors[135]. The afferent inputs to the VTA controlling sleep are not clearly defined, but we demonstrate dense Nts inputs from the POA to the VTA, and the POA is an established regulator of sleep-wake behaviors (see [136] for review). GABAergic neurons in the ventrolateral POA promote induction and maintenance of sleep[137,138] and project to the VTA[139], though it remains to be determined if these neurons co-express Nts. Nts has, however, been implicated in sleeping behavior. For example, central administration of Nts alters sleeping patterns[7,140] and NtsR1 knockout mice have altered baseline sleeping behavior and disrupted response to sleep deprivation[6]. Thus, the large number of Nts inputs to the VTA from the LPO and MPO therefore could potentially play a role in mediating sleep, and may be useful targets to treat the sleep disturbances that commonly co-occur in many neuropsychiatric diseases.

Functional Implications of Nts Afferents to the VTA from the LHA: The LHA provided a large number of Nts afferents to the VTA of mice, consistent with prior tracing studies from mice[141] and rats[83]. The LHA is a hub for coordinating peripheral energy status with motivated behaviors such as feeding and locomotor activity that impact body weight (see [5] for review). Indeed, pharmacological Nts treatment reduces feeding and increases locomotor activity in a NtsR1-dependent manner[52,53,77,78,142-144], and these effects are mediated, in part, by LHA Nts neurons that project to the VTA[39,87,88]. The LHA Nts neurons are molecularly distinct from other orexigenic neuropeptide-containing populations such as orexin (OX) and melanin-concentrating hormone (MCH) neurons[86], and instead are selectively activated by peripheral cues that suppress feeding such as leptin, inflammation, and dehydration[88,145,146]. Furthermore, loss of leptin receptor signaling from LHA Nts neurons leads to hypoactivity and weight gain[88]. Given that LHA Nts neurons respond to cues that suppress feeding and project directly to the VTA, we

hypothesize that they coordinate peripheral energy status with feeding behavior and energy expenditure, a process that becomes disrupted in obesity and eating disorders. Since stimulation of LHA fibers projecting to the VTA and Nts administration into the VTA are both rewarding[50,59,62,147], LHA Nts afferents may also regulate other motivated via the VTA. For example, some LHA neurons that project to the VTA are activated by acute amphetamine exposure[148], expression of morphine CPP[149], and cue-induced reinstatement of cocaine[150]. Going forward, it will therefore be important to define the specific contributions of LHA Nts inputs to the VTA to Nts-mediated behaviors, and whether they modify consumption of both natural and pharmacologic rewards.

Functional Implications of Nts Afferents to the VTA from the NA: The NAsh provides substantial Nts projections to the VTA similar to the magnitude from the MPO and LPO. NAsh Nts neurons likely overlap with the GABAergic medium spiny neurons in this region that project to the VTA[151,152], which are thought to comprise a feedback loop to regulate the activity of VTA DA neurons[153,154]. For example, NA medium spiny neuron projections to VTA GABA neurons cause dis-inhibition of neighboring DA neurons, which contributes to the reinforcing effects of cocaine[155]. NA afferents to the VTA are also preferentially activated during acute amphetamine exposure[148] and cue-induced reinstatement of cocaine[150]. Together these data indicate that NA projections to the VTA may be involved in drug-seeking behavior, thus NAsh Nts projections are anatomically positioned to modify intake of pharmacological rewards. While many studies implicate roles for central or systemic Nts treatment in modifying drug-intake, it remains unclear what part of these effects are mediated specifically by Nts release into the VTA (see [8] for review). Injection of Nts into the VTA does not alter the acute response to amphetamine[56], but may be required for the expression of amphetamine sensitization[58]. Thus, although administration of Nts to the VTA is rewarding, it is unclear whether NAsh Nts inputs specifically modulate the strong reinforcing properties of abusive drugs or natural rewards.

Conclusions: Collectively, our work defines the central circuits capable of providing endogenous Nts to the VTA in mice, which are summarized in Fig. 11. Identification of these Nts afferents to the VTA also suggests specific mechanisms that presumably underlie the ability of Nts to modify feeding, sleeping, and social behavior. These data will be useful to direct the application of optogenetic and/or chemogenetic strategies to selectively modulate the activity of site-specified Nts populations, and thereby to define their contributions to behavior and physiology. One limitation of our present work is that it identifies Nts neurons that project to the VTA in general, but does not reveal which specific VTA neurons are targets of Nts signaling (e.g. DAergic, GABAergic, and/or glutamatergic neurons). Since it is now appreciated that VTA neuronal subpopulations control specific motivated behaviors and their reinforcing or aversive valence, it will be crucial to establish how Nts neurons mechanistically engage VTA neurons to fully understand their role in behavior. Indeed, given that Nts inputs from the LHA and POA appear to differentially modify motivated behaviors, there may be distributed Nts circuits and Nts mechanisms to appropriately coordinate environmental stimuli with feeding, arousal and/or social interaction responses. Modulation of specific Nts afferents to the VTA may therefore be useful to normalize maladaptive behaviors associated with neuropsychiatric diseases.

2.5 Methods

Animals: Mice were bred and housed in a 12h light/12h dark cycle and cared for by Campus Animal Resources (CAR) at Michigan State University. Animals had *ad lib* access to chow (Teklad 7913) and water. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Nts^{cre} mice [88] [Jackson stock ♯ 017525], were bred to wild-type C57/Bl6 mice for seven generations to obtain fully backcrossed animals. To visualize Nts neurons, heterozygous *Nts*^{cre}

mice were crossed with homozygous *Rosa26*$^{EGFP\text{-}L10a}$ mice [90], and progeny heterozygous for both *Nts*^{cre} and *Rosa26*$^{EGFP\text{-}L10a}$ alleles were studied (referred to as *Nts*^{cre}*;GFP*). Genotyping was performed using standard PCR using the following primer sequences: *Nts*^{cre}*:* common forward: 5' ATA GGC TGC TGA ACC AGG AA, Cre reverse: 5' CCA AAA GAC GGC AAT ATG GT and WT reverse: 5' CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26*$^{EGFP\text{-}L10a}$: mutant forward: 5' TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5' GAG GGG AGT GTT GCA ATA CC and common reverse: 5' CAG ATG ACT ACC TAT CCT CCC. Adult male and female *Nts*^{Cre}*;GFP* mice were used for all studies.

Surgery: Adult *Nts*^{Cre}*;GFP* mice received a pre-surgical injection of carprofen (5mg/kg *s.c.*) and were anesthetized with 3-4% isoflurane/O_2 in an induction chamber before being placed in a stereotaxic frame (Kopf). Under 1-2% isoflurane, access holes were drilled in the skull and a guide cannula with stylet (PlasticsOne) was used to deliver 15 nL of FG unilaterally into the VTA in accordance with the atlas of Paxinos and Franklin[156] (A/P: -3.2mm, M/L: -0.48mm, D/V: -4.6mm from bregma). Animals were included in the study if 1) the FG injection was targeted to and confined within the VTA and 2) FG-labeled neurons were observed in a pattern consistent with mouse VTA afferents based on the work of Watabe-Uchida *et al.* and Faget *et al.*[93,96]. Out of 10 FG-injected *Nts*^{Cre}*;GFP* mice, 5 were excluded from analysis because the injection was targeted lateral or dorsal to the VTA and/or the pattern of FG-labeled neurons was more consistent with SN rather than VTA afferents (see Fig. 3 for verification).

Perfusions and Immunohistochemistry: Seven days after surgery, FG-injected *Nts*^{cre}*;GFP* mice were deeply anesthetized with sodium pentobarbital, transcardially perfused with 10% formalin and brains were post-fixed for 24 hours. After dehydration with 30% sucrose the brains were coronally sectioned (30 μm) using a freezing microtome (Leica). Each brain was sectioned into four separate but equally represented series. Immunofluorescence was

performed as previously described[88]. Sections were incubated with chicken anti-GFP (1:2000, Abcam) and mouse anti-tyrosine hydroxylase (TH) (1:1000, Millipore) or rabbit anti-fluorogold (1:500, Fluorochrome).

Imaging and Quantification: Brain sections were analyzed using an Olympus BX53 fluorescence microscope outfitted with FITC and Texas Red filters. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe). Each image was assigned a bregma coordinate based on the mouse brain atlas of Paxinos and Franklin[156]. For each brain area of interest, images from the 5 well-targeted animals were organized by bregma coordinate and placed side-by-side with images of *Nts* ISH from the Allen Brain Project[100]. For quantification of Nts neurons in the VTA, four representative coronal sections across similar bregma coordinates from each animal were assessed for total number of Nts neurons and colocalization with TH. Images were viewed and quantified in Photoshop CS6 (Adobe). To estimate the total number of Nts neurons that project to the VTA, 10x images of every section from FG-injected *Nts^{Cre};GFP* mice were analyzed for the number the neurons that colocalized with Nts and FG. To improve visualization of FG+ neurons, a contrast mask of +100 was applied to each image for quantification. To ensure that each area was sufficiently represented in each animal, the total span over which a brain area was counted in mm (determined by difference between the two furthest bregma coordinates) and the distance was divided by the total number of sections analyzed. Because 30µm brain sections were collected in four separate series, each section represented approximately 0.12 mm of tissue; thus the average mm per section analyzed for each brain area was kept as close to 0.12 as possible to ensure that the entire region was accounted for in the rostrocaudal axis. Animals that did not have enough sections to meet these criteria for a given region were excluded from quantification. To facilitate clarity of neuronal features, all figures

depicting FG+ neurons have been digitally enhanced in Photoshop to improve contrast and, in some cases, brightness.

APPENDIX

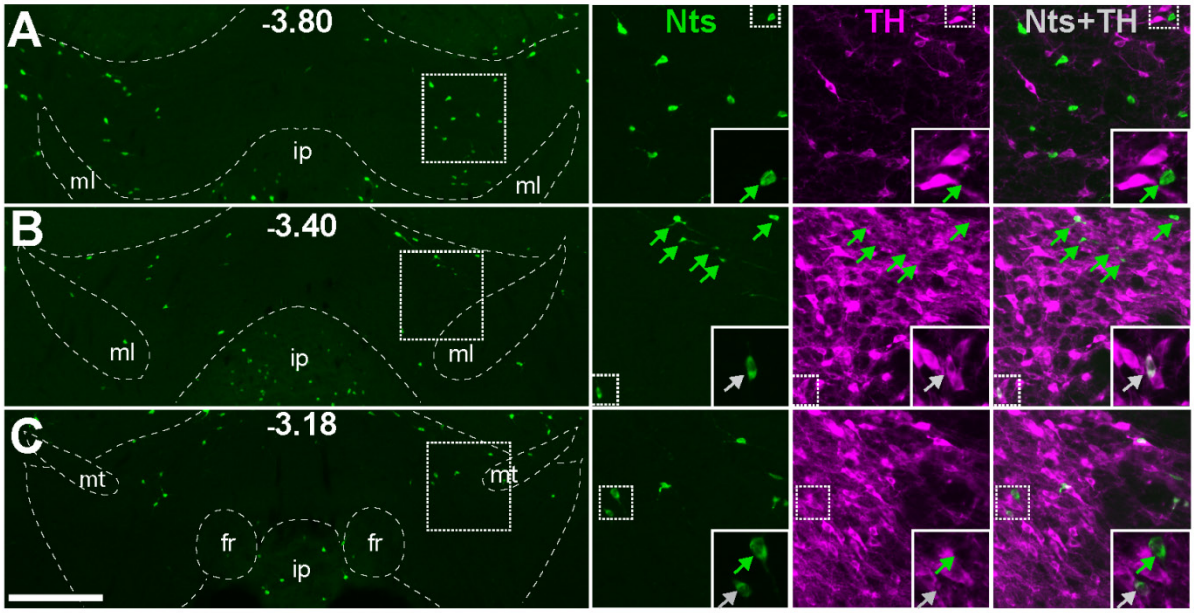

Figure 2. Local Nts expression in the VTA. *Nts^{Cre};GFP* mice (n = 6) were used to determine the number and distribution of VTA Nts neurons and whether or not they co-express TH. **A,B,C)** Representative images of GFP-identified Nts neurons across three different bregma coordinates in the VTA of *Nts^{Cre};GFP* mice (scale bar = 200um). Insets highlight individual Nts neurons and the presence or absence of colocalization with TH (gray arrows=TH+ Nts neurons, white arrows=TH-negative Nts neurons). *ml=medial lemniscus, ip=interpeduncular nucleus, fr=fasciculus retroflexus, mt=mammillothalamic tract.*

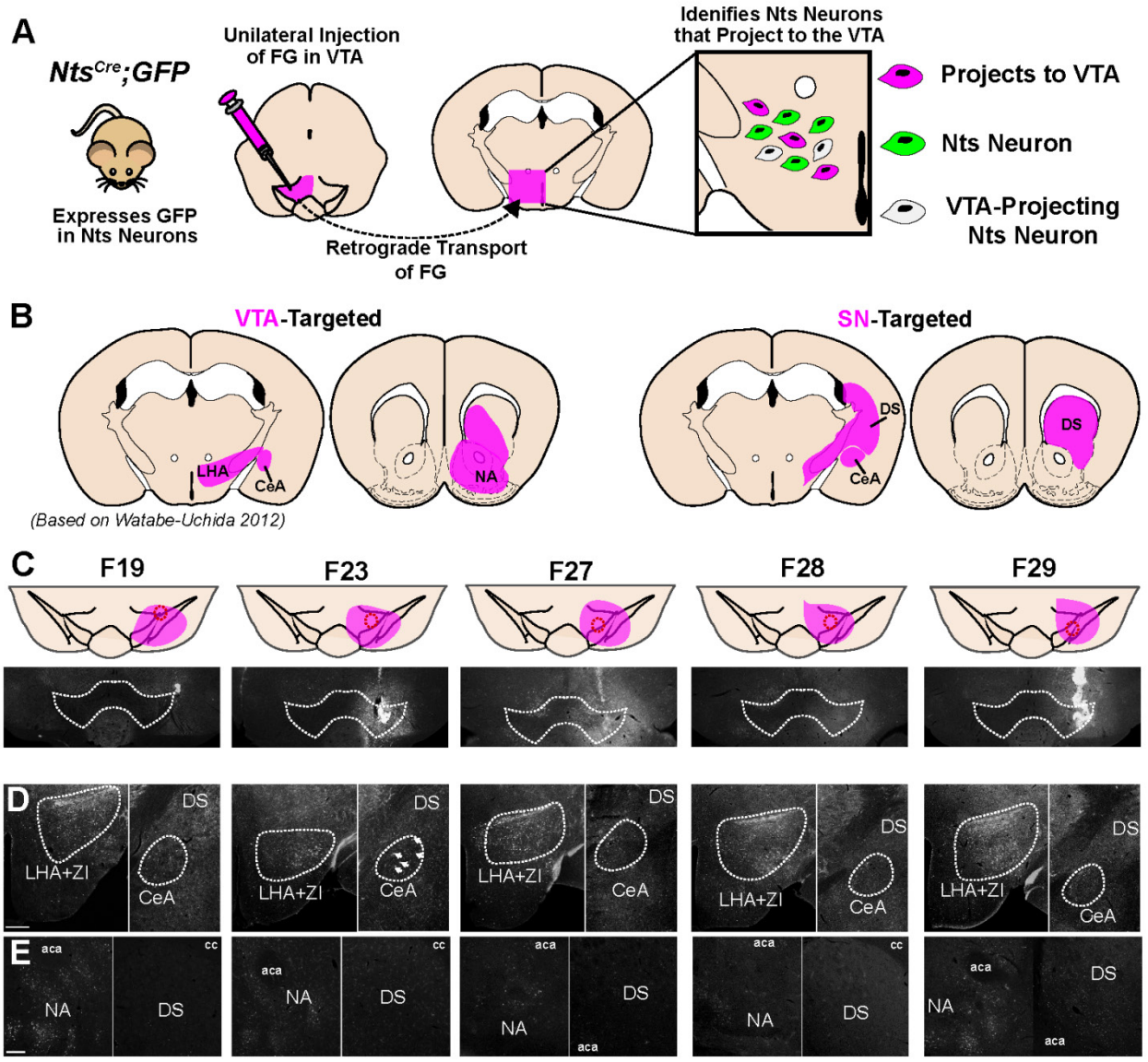

Figure 3. Validation of VTA targeting in *Nts^{Cre};GFP* mice injected unilaterally with FG. **A)** *Nts^{Cre};GFP* reporter mice were injected unilaterally in the VTA with FG allowing for simultaneous visualization of neurons that project to the VTA (FG positive neurons), Nts neurons (GFP positive neurons) and Nts afferents to the VTA (GFP/FG+ double-positive neurons). **B)** Expected afferent patterns to the VTA or SN based on Watabe-Uchida *et al.*[93]. **C)** Midbrain images of the five VTA-targeted *Nts^{Cre};GFP* mice included in the final analysis. Red dotted-line circles indicate the injection sites while the spread of FG is shown in white. **D**) FG-labeled cell bodies of VTA-targeted animals are confined to the LHA, ZI, CeA, and absent from the DS. Scale bar=200μm. **E)** FG-labeled neurons of VTA-targeted animals are located preferentially in the ventral, not dorsal striatum. Scale bar=100μm.

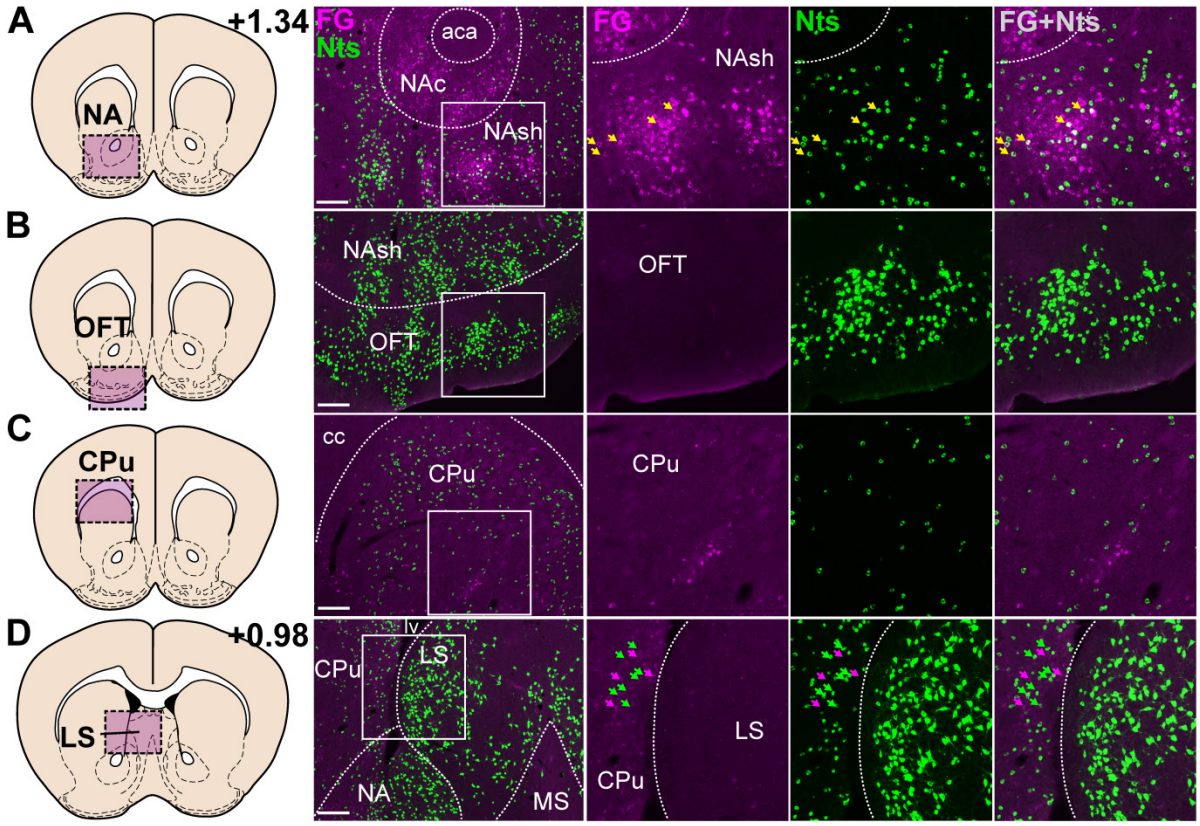

Figure 4. The NA shell contains clusters of Nts neurons that project to the VTA. A) Many Nts/FG+ neurons were found in the NAsh (yellow arrows), but not NAc. **B)** Nts expression in the OFT, an area that does not provide significant VTA input and hence few FG+ neurons were observed. **C)** Representative expression of Nts and FG in the CPu shows little to no colocalization. **D)** Nts neurons were numerous in the LS, an area that does not provide substantial input to the VTA. Green or purple arrows represent non-colocalized GFP or FG+ neurons in the neighboring CPu. Scale bar=100uM. *NAc=nucleus accumbens core, NAsh=nucleus accumbens shell, OFT=olfactory tubercle, CPu=caudate/putamen, LS=lateral septum, MS=medial septum, lv=lateral ventricle, cc=corpus callosum, aca=anterior commissure.*

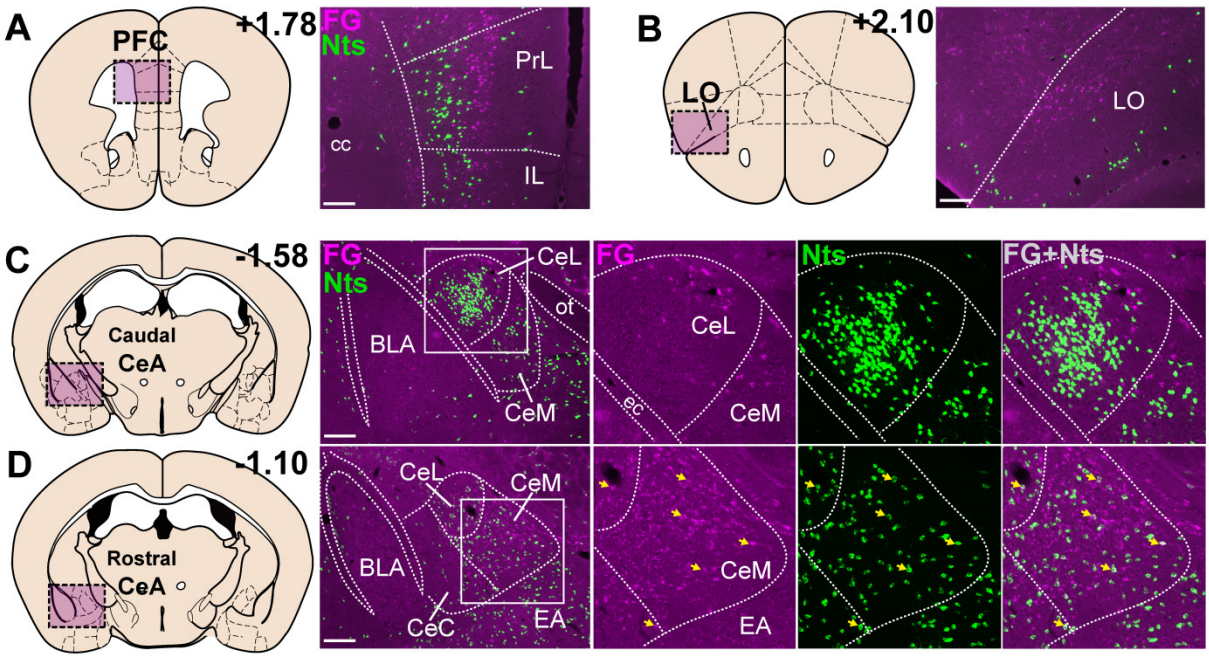

Figure 5. Cortical Nts inputs to the VTA originate in the CeA but not neocortex. Representative images show the absence of FG and Nts co-labeling in the **A)** PFC and **B)** LO. **C)** A dense population of Nts neurons resided in the lateral division of the caudal CeA, but these neurons were unlikely to project to the VTA. **D)** Some Nts neurons in the medial CeA colocalized with FG (yellow arrows). Scale bar=100uM. *PFC= prefrontal cortex, CeA=central amygdala, CeM=medial division of CeA, CeL=lateral division of CeA, CeC=central division of central amygdala, BLA=basolateral amygdala, ot=optic tract, EA=extended amygdala, PrL=prelimbic cortex, IL=infralimbic cortex, LO=lateral orbital cortex, ec=external capsule.*

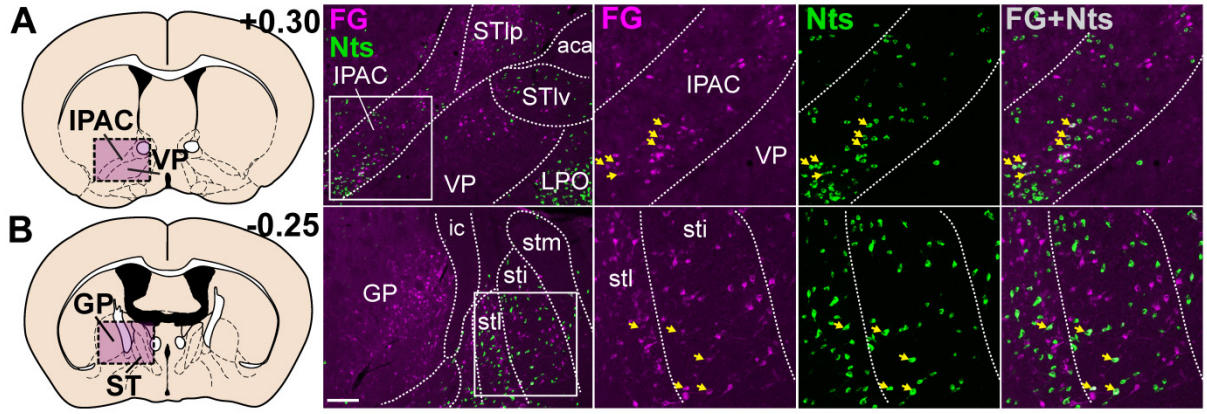

Figure. 6. Nts neurons in the lPAC, pallidum, and ST that project to the VTA. A) Nts/FG+ neurons were observed in the lateral lPAC (yellow arrows) while the adjacent VP contained low numbers of Nts neurons. **B)** Nts/FG+ neurons were observed in the ST (yellow arrows), but not GP. Scale bar=100uM. *STlp=lateral posterior division of stria terminalis, STlv= lateral ventral division of stria terminalis, stm=medial portion of bed of stria terminalis, sti=intermediate division of bed of stria terminalis, stl=lateral division of bed of stria terminalis, ic=internal capsule, GP=globus pallidus, VP=ventral pallidum, LPO=lateral preoptic area, aca=anterior commissure.*

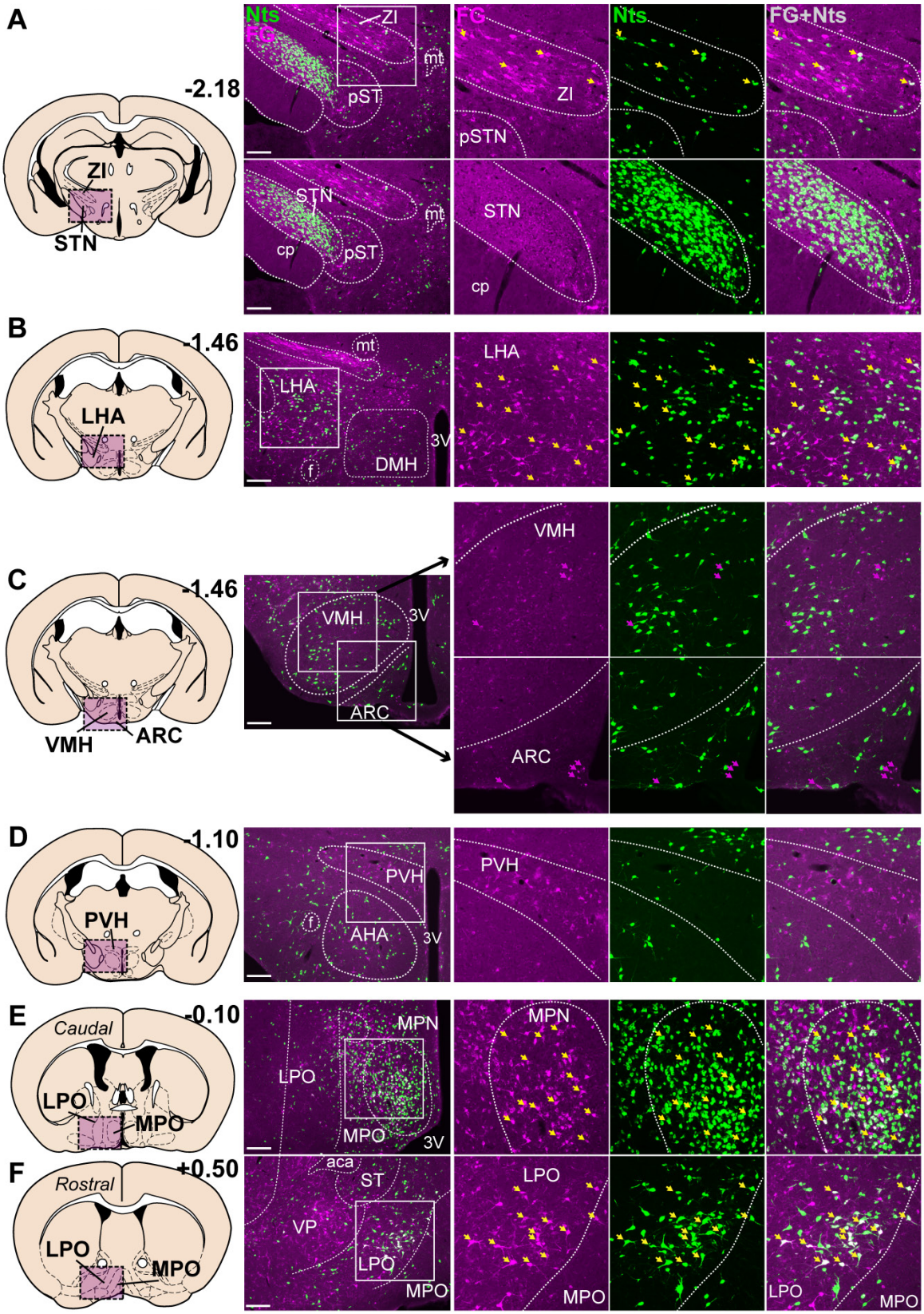

Figure 7. Sub-regions of the hypothalamus provide Nts afferents to the VTA.

Figure 7 (*cont'd*). A) Caudal section of the hypothalamus showing Nts/FG+ neurons in the ZI (top panel, yellow arrows) but not the STN (bottom panel). **B)** The LHA contained a large number of Nts/FG+ neurons (yellow arrows). **C)** The VMH (top panel) and ARC (bottom panels) did not provide many Nts afferents to the VTA. Purple arrows indicate VTA afferents that did not express GFP. **D)** The PVH contained minimal Nts neurons and was not a major source of Nts input to the VTA. **E)** A dense Nts population resided in the caudal MPO and many of these were Nts/FG+ neurons that projected to the VTA (yellow arrows). **F)** Numerous Nts/FG+ neurons were also observed in the rostral LPO (yellow arrows). Scale bars=100uM. *ZI=zona incerta, pSTN=para subthalamic nucleus, cp=cerebral peduncle, mt=mammillothalamic tract, STN=subthalamic nucleus, LHA=lateral hypothalamic area, DMH=dorsomedial hypothalamic area, 3V=third ventricle, f=fornix, VMH=ventromedial hypothalamus, ARC=arcuate nucleus, PVH=paraventricular nucleus, AHA=anterior hypothalamic area, LPO=lateral preoptic area, MPO=medial preoptic area, MPN=medial preoptic nucleus.*

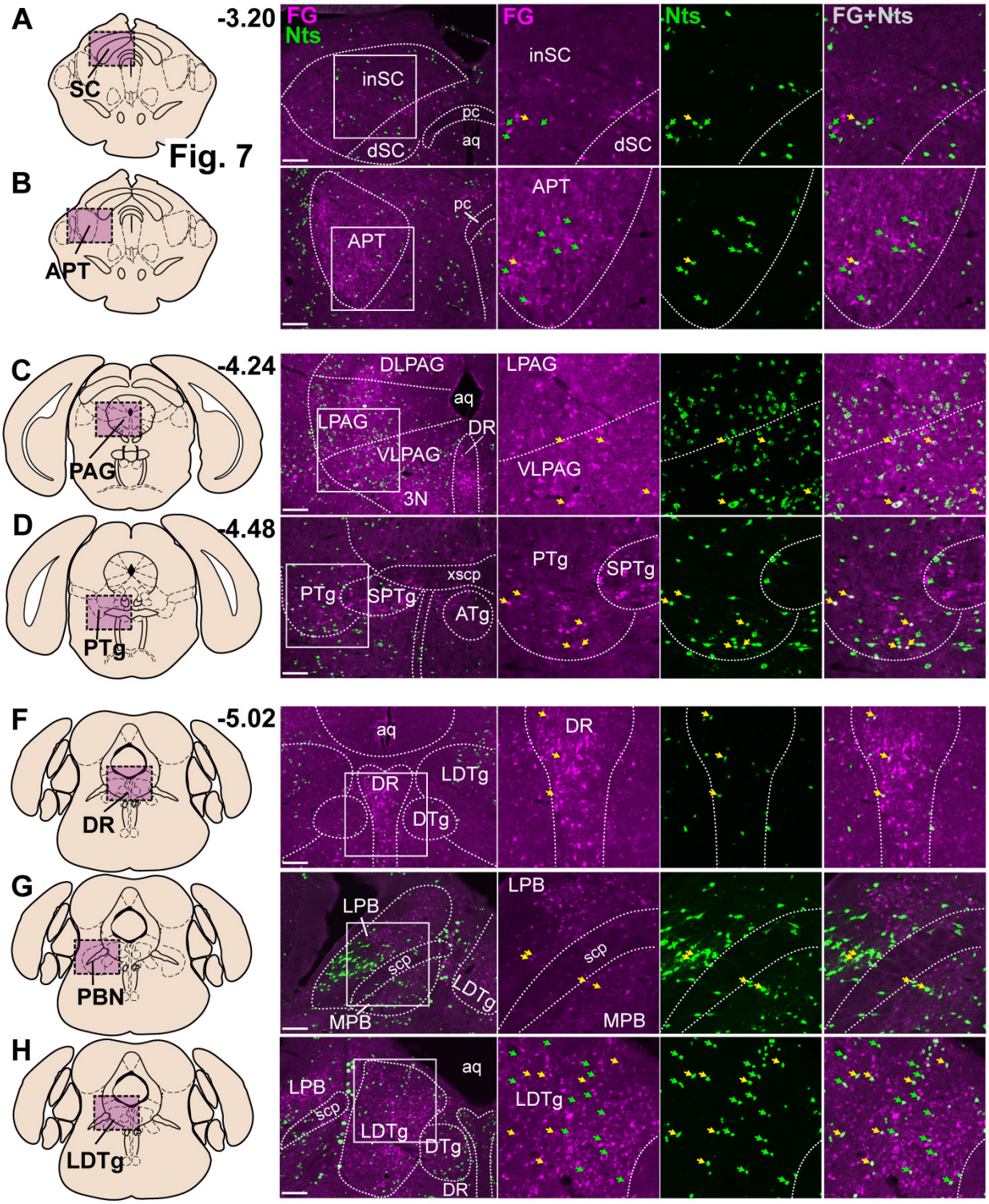

Figure 8. Brainstem Nts inputs to the VTA. A small number of Nts/FG+ neurons were detected in the **A)** superior colliculus (SC) and **B)** anterior pretectal nucleus (APTN).

Figure 8 (*cont'd*). Representative images show occasional FG/Nts+ neurons observed in the **C)** PAG, **D)** PTg, **E)** DR, **F)** PBN, and **G)** LDTg. Scale bar=100uM. *SC=superior colliculus, iSC=inferior superior colliculus, dSC=dorsal superior colliculus, pc=posterior commissure, aq=cerebral aqueduct, APT=anterior pretectal nucleus, PAG=periaqueductal gray, DLPAG=dorsolateral PAG, LPAG=lateral PAG, VLPAG=ventrolateral PAG, 3N=oculomotor nucleus, DR=dorsal raphe, PTg=peduncolopontine tegmentum, SPTg=subpenduncular tegmental nucleus, xscp=decussation of superior cerebellar peduncle, ATg=anterior tegmental nucleus, LDTg=laterodorsal tegmental nucleus, scp=superior cerebellar peduncle, DTg=dorsal tegmental nucleus, MPBN=medial parabrachial nucleus, LPBN=lateral parabrachial nucleus.*

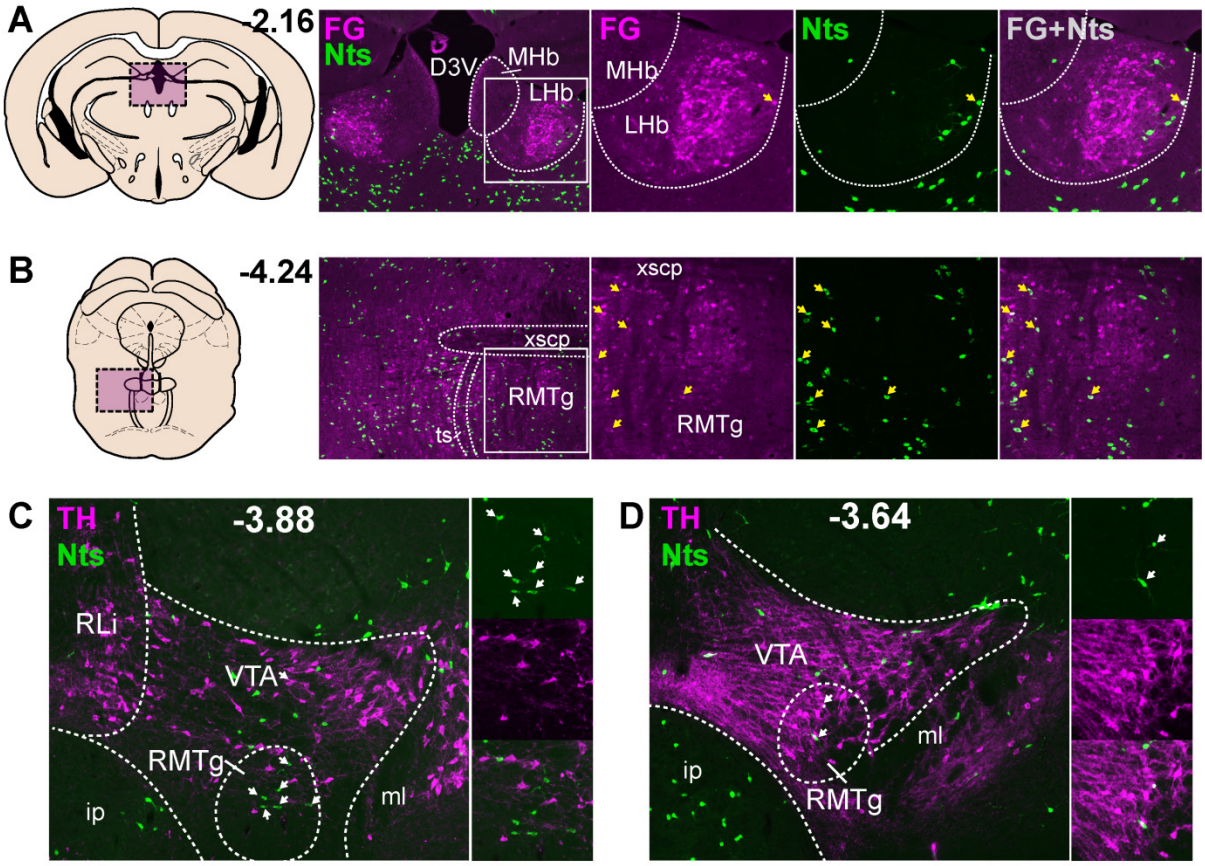

Figure 9. Nts-ergic VTA inputs from the LHb and RMTg. A) The LHb contained only occasional colocalized neurons (yellow arrow). **B)** Some Nts/FG+ neurons were detected in the caudal (yellow arrows). **C, D)** Images of the rostral RMTg in uninjected *Nts^{Cre};GFP* mice showing TH-negative Nts neurons (white arrows). *D3V=dorsal third ventricle, MHb=medial habenula, LHb=lateral habenula, xscp=decussation of cerbellar peduncle, ts=tectospinal tract, RLi=raphe linear nucleus, ip=interpeduncular nucleus, ml=medial lemniscus.*

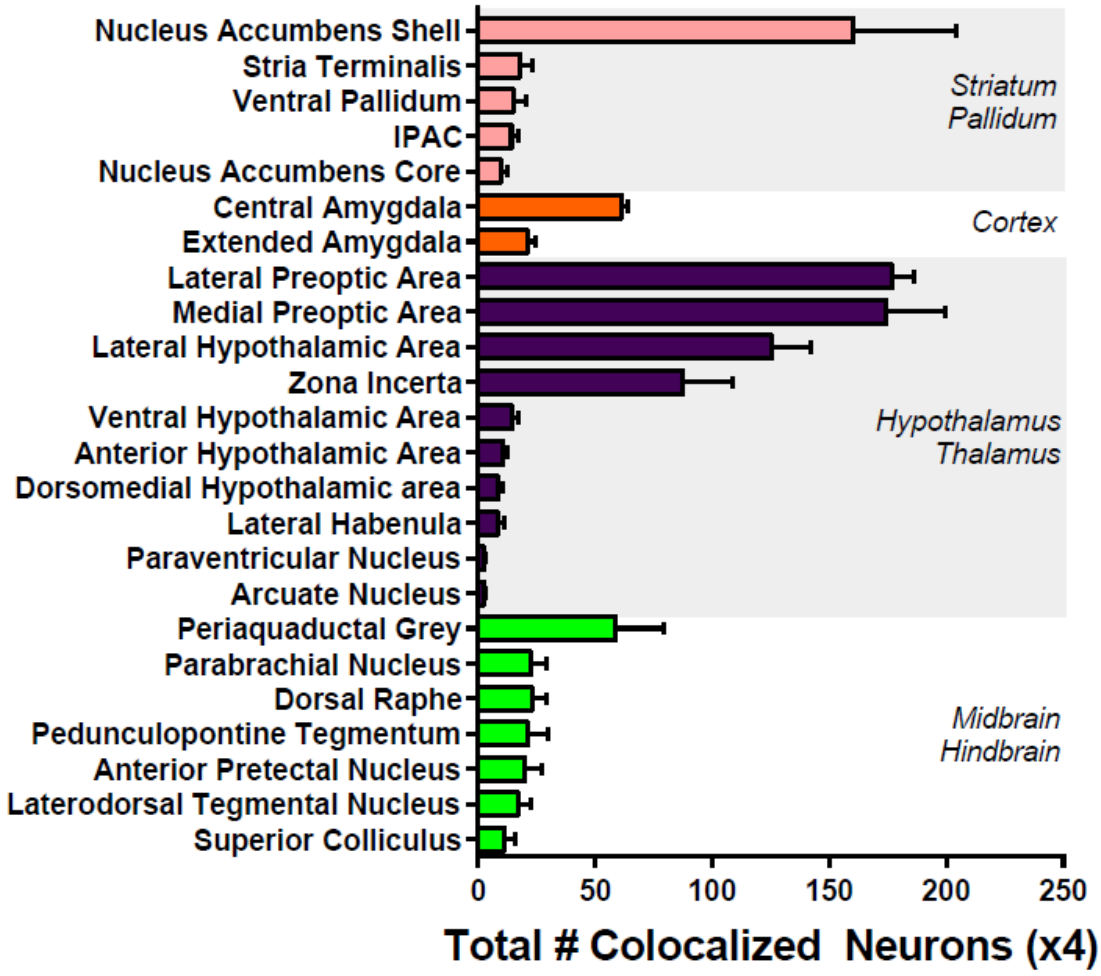

Figure 10. Quantification of Nts-expressing afferents to the VTA by anatomical sub-region. Cell bodies colocalizing with GFP and FG were quantified by sampling every fourth coronal section, thus the bars represent approximately one quarter of the total number of neurons in each region. Areas that contained ≤1 colocalized neuron per section were not included in the quantification.

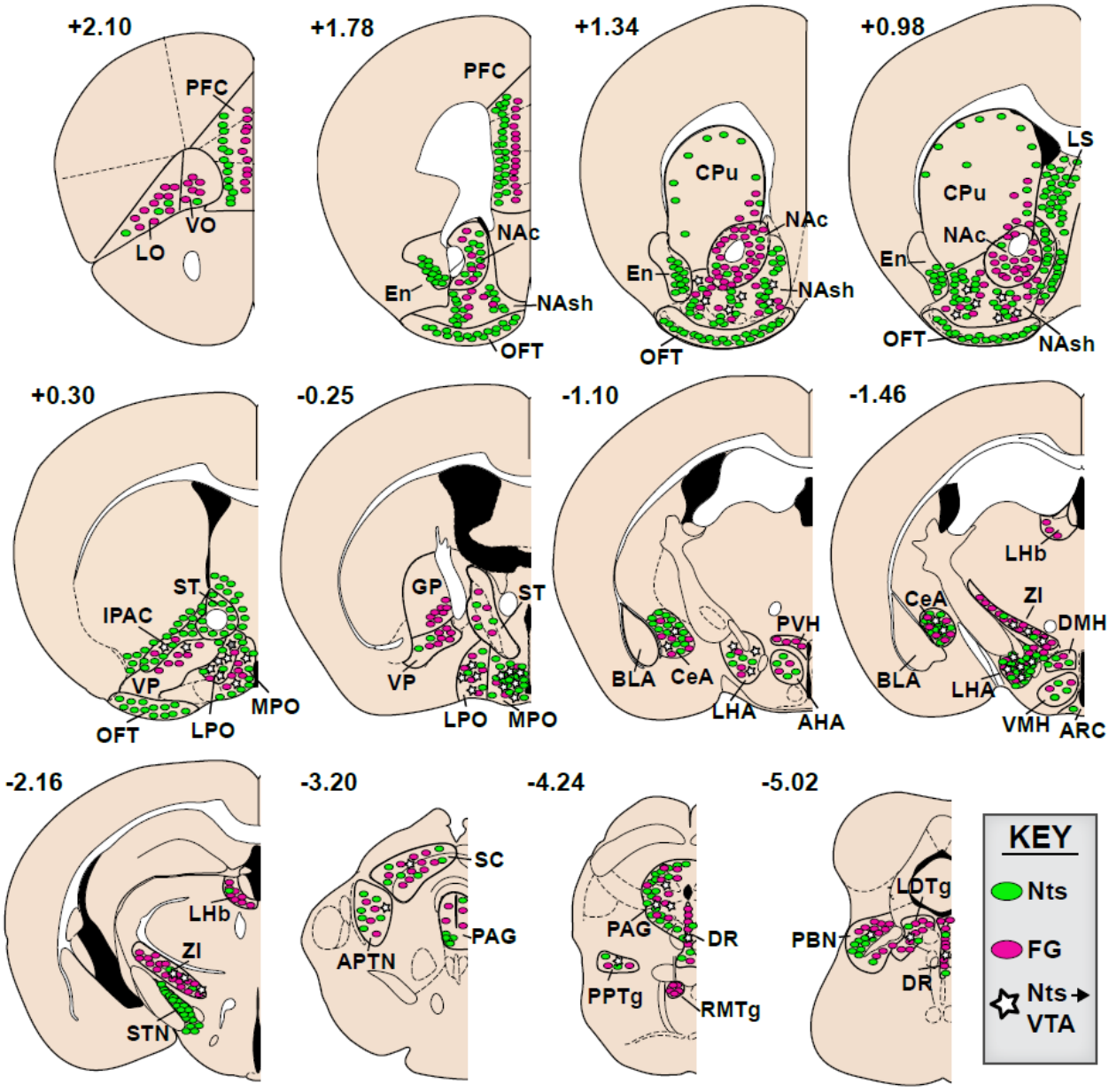

Figure 11. Schematic illustration of Nts afferents to the VTA. Distribution patterns of Nts neurons and VTA afferents at different bregma coordinates. Green ovals represent GFP-labeled neurons, pink ovals represent FG-labeled neurons, and white stars indicate colocalized Nts/FG+ neurons. Only the sub-regions included in analysis are outlined in black and labeled, and blank regions do not necessarily indicate lack of GFP or FG.

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Chapter 3. Identification of Neurotensin Receptor Expressing Cells in the Ventral Tegmental Area Across the Lifespan

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The is a modified version of a manuscript under revision.

3.1 Abstract

Neurotensin (Nts) promotes activation of dopamine (DA) neurons in the ventral tegmental area (VTA) via incompletely understood mechanisms. Nts can signal via the G-protein coupled neurotensin receptors 1 and 2 (NtsR1 and NtsR2), but the lack of methods to detect NtsR1 and NtsR2-expressing cells has limited mechanistic understanding of Nts action. To overcome this challenge, we generated dual recombinase mice that express FlpO-dependent Cre recombinase in NtsR1 or NtsR2 cells. This strategy permitted temporal control over recombination, such that we could identify NtsR1 or NtsR2-expressing cells and determine if their distributions differed between the developing and adult brain. Using this system we found that NtsR1 is transiently expressed in nearly all DA neurons and in many non-DA neurons in the VTA during development. However, NtsR1 expression is more restricted within the adult brain, where only two thirds of VTA DA neurons expressed NtsR1. By contrast, NtsR2 expression remains constant throughout lifespan but it is predominantly expressed within glia. Anterograde tract tracing revealed that NtsR1 is selectively expressed by mesolimbic, not mesocortical DA neurons, thus VTA NtsR1 neurons are a projection-specified and functionally unique subset of VTA DA neurons. Collectively, this work reveals a cellular mechanism by which Nts can directly engage NtsR1-expressing DA neurons to modify DA signaling. Going forward, the dual recombinase strategy developed here will be useful to selectively modulate NtsR1 and NtsR2-expressing cells, and to parse their contributions to Nts-mediated behaviors.

3.2 Introduction

The neuropeptide, neurotensin (Nts), is expressed throughout the central nervous system and has been linked to numerous physiologic and behavioral processes including regulation of feeding, body temperature, nociception, emotional state, and sleep[1-5]. While the precise neural circuits by which Nts acts to modify behavior remain undetermined, it is well-established that Nts modulates dopamine (DA) transmission. Nts signaling may therefore be a target of interest for the treatment of diseases linked with altered DA signaling, including Parkinson's disease, schizophrenia, drug addiction, and obesity[5,6].

Nts signals via the G-protein coupled neurotensin receptors 1 and 2 (NtsR1 and NtsR2) which are both expressed in the brain and have been implicated in Nts-mediated behaviors[7-10]. Nts also binds the intracellular receptor, NtsR3/sortilin, which may regulate recycling and sorting of Nts[3,11]. Studies using pharmacological antagonists and mice null for NtsR1 or NtsR2 suggest that each receptor isoform mediates distinct aspects of physiology, and hence that they may be expressed on different cell types. The lack of methods to detect and manipulate NtsR1 or NtsR2-expressing cells, however, has limited understanding of the cellular mechanisms by which Nts regulates behavior. NtsR1 was the first NtsR identified and has a 10-30 fold higher affinity for Nts compared to NtsR2[7-10]. Nts binding to NtsR1 activates G_q-coupled induction of phospholipase C and intracellular Ca^{++} release[7,8,12]. The signaling mechanism of NtsR2 is less clear, and both G_q and G_i-coupled mechanisms have been reported[9,13]. *In situ* hybridization (ISH) and autoradiography methods to detect NtsR1 indicate that it is expressed robustly within the ventral tegmental area (VTA) and substantia (SN) of adult animals[14-17]. Similar techniques revealed diffuse expression of NtsR2 throughout the brain that may be within both neurons and glia[18-20], with the highest levels of expression restricted to the cerebellum, hippocampus, and periaqueductal gray (PAG)[9,19,21]. Interestingly, the expression patterns of NtsR1 and NtsR2 in

the brain may vary with age. For example, central NtsR1 is transiently upregulated during gestation and peaks during the first week of life, but is subsequently downregulated as animals reach maturity, with high levels persisting in select brain regions including the VTA and SN[22,23]. By contrast, NtsR2 expression is low at birth but gradually increases with age, peaking in adulthood[21,22]. Taken together, these data indicate that NtsR1 and NtsR2 have distinct expression patterns that vary across the lifespan, and may be found on different cell types within the nervous system.

Given the different sites and distribution of NtsR1 and NtsR2 expression throughout lifespan, each receptor isoform may regulate distinct aspects of developmental and adult physiology. Indeed, previous work demonstrates that central Nts promotes DA release, locomotor activity, hypothermia, anorexia, and reward via NtsR1[1,24-29], whereas NtsR2 may confer the pain-reducing effects of Nts[1,3,30,31]. However, the evidence for distinct roles of NtsR1 and NtsR2 is not entirely consistent and interpretation of the data is complicated by methodological limitations. For example, the commonly used NtsR1-selective antagonist SR48692 also acts as an agonist at NtsR2[13,32,33], while a potential compound to selectively antagonize NtsR2 has only recently been developed[3,34]. NtsR1 and NtsR2 knockout mice have also been used to examine the specific roles of each receptor, but developmental deletion in these models may lead to compensatory changes that mask normal action of the Nts system. For example, it has been suggested that NtsR1 or NtsR2 null mice have increased expression of the remaining receptor isoform[26,35] and exhibit alterations in DA signaling and locomotor activity that misrepresent the functional roles of the NtsR receptor signaling[1,24,26,35,36]. Finally, the lack of immunohistochemical reagents to detect NtsR1 or NtsR2 has impeded determination of the cells and circuits that mediate Nts action. Thus, while NtsR-selective pharmacologic agents and knockout models have added to understanding of central Nts action, developing

methods to visualize and manipulate select NtsR1 or NtsR2 populations *in vivo* is essential to deciphering the neural circuits and physiology regulated by each receptor.

To address this challenge, we developed dual recombinase knock-in mouse models in which FlpO is required to induce IRES-Cre in cells that express NtsR1 or NtsR2. Cre-mediated recombination can then be used to induce fluorescent reporters in these cells to permit their detection, and indeed Cre-driver lines have proven to be reliable reagents to identify genetically specified cell populations[37-39]. A limitation of mouse models that constitutively express Cre is that recombination-mediated labeling occurs whenever Cre is expressed and persists thereafter. This may confound interpretation in cases where gene-induced Cre expression changes over lifespan, as has been suggested to occur for NtsRs (with extensive expression during development but more restricted expression in the adult brain[22,23]). We thus engineered FlpO-dependent Cre expression in NtsR1 and NtsR2 cells, allowing for temporal control over recombination by inducing FlpO expression at defined time points (either embryogenesis or adulthood). Given the well-established description of Nts as a modulator of DA signaling, but the lack of understanding of which VTA cells mediate it, we used these mice to define the cellular distribution of NtsR1 and NtsR2 within the VTA.

3.3 Results

Dual Recombinase Strategy to Label NtsR1 and NtsR2 Expressing Cells: To identify NtsR1 or NtsR2 cells “on command”, we generated knock-in mouse models that express Cre in NtsR1 or NtsR2 cells only after FlpO-mediated recombination. To do this, we inserted an *frt*-flanked NEO cassette upstream of an IRES-Cre sequence and cloned it into the non-coding region of the *Ntsr1* or *Ntsr2* genomic sequences; we refer to these as

NtsR1$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice (Fig. 12). The *frt*-flanked NEO cassette blocks Cre expression unless NEO is removed, thus *NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice lack Cre expression until exposure to FlpO.

To reveal cells that express NtsR1 and NtsR2 during development, we crossed *NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice to a FlpO deleter line, producing progeny that lack the *frt*-flanked NEO cassette and thus produce Cre whenever NtsR1 or NtsR2 is transcribed throughout the lifespan (*NtsR1*$^{\Delta NEO\text{-}Cre}$ and *NtsR2*$^{\Delta NEO\text{-}Cre}$ mice). The *NtsR1*$^{\Delta NEO\text{-}Cre}$ and *NtsR2*$^{\Delta NEO\text{-}Cre}$ mice were subsequently bred to a Cre-inducible eGFP-L10a reporter line; in progeny heterozygous for each allele, any cell that expresses NtsR1 or NtsR2 will undergo Cre-mediated recombination to express GFP. Notably, the recombination is permanent, so GFP labeling persists even in cells that cease to express NtsR1 or NtsR2. This model enables us to visualize any cells that expressed NtsR1 or NtsR2 from conception onward, and we refer to these as *NtsR1*^{Dev}*;GFP* and *NtsR2*^{Dev}*;GFP* mice (Fig. 12A).

To study the adult expression pattern of NtsR1 and NtsR2, we bred *NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice to the Cre-inducible eGFP-L10a reporter. In this case, the progeny (*NtsR1*$^{NEO\text{-}Cre}$*;GFP* and *NtsR2*$^{NEO\text{-}Cre}$*;GFP*) carry the *GFP* allele, but no reporter is expressed because the *frt*-flanked NEO cassette suppresses Cre expression. These mice were maintained in our colony until they reached adulthood, then were injected with an adenovirus expressing FlpO recombinase into the lateral ventricles to permit FlpO expression throughout the adult brain. FlpO excises the *frt*-flanked NEO, which permits Cre-mediated GFP expression only in cells that actively express NtsR1 or NtsR2, allowing us to visualize the adult expression pattern of NtsR1 and NtsR2 (*NtsR1*^{Adult}*;GFP* and *NtsR2*^{Adult}*;GFP*) (Fig. 12B).

Distribution and Morphology of NtsR1 and NtsR2 in the VTA: Previously, identification of NtsR1 and NtsR2 neurons has been challenging given the lack of reliable immunohistochemical reagents to visualize them. The dual recombinase mouse models we generated, however, enabled us to visualize the distributions of NtsR1 and NtsR2 in the VTA, and whether they differed between developmental and adult stages. First, analysis of *NtsR1^{Dev};GFP* mice revealed a wide-spread, dense population of GFP+ neurons in the VTA (Fig. 13A) and throughout the brain (data not shown). Despite many GFP-labeled neurons, we ruled out the possibility of ectopic expression via lack of GFP in regions that are thought to have minimal NtsR1 expression at any point in life, including the interpeduncular nucleus (ip)[40] (see ip in Fig. 13A). By contrast, *NtsR1^{Adult};GFP* mice had far fewer GFP+ neurons in general and were primarily restricted to the VTA and SN (Fig. 13B). These findings are consistent with previous work showing that central NtsR1 expression peaks during gestation, but subsequently decreases and only remains prominent within the midbrain of adult rodents[22,23].

Analysis of NtsR2-reporter mice revealed two morphologically distinct populations of GFP-labeled cells in the VTA. A few GFP+ cells displayed clear neuronal features (Fig. 13C, white arrows) but the vast majority of GFP-labeled cells in both developmental and adult models were detected in cells with diffuse, stellate morphology indicative of glial cells (Fig. 13C). This finding supports previous work suggesting that NtsR2 is expressed on astrocytes[20,41,42]. Neuronal GFP expression was sparse in both *NtsR2^{Dev};GFP* and *NtsR2^{Adult};GFP* models but slightly more neurons were detected in *NtsR2^{Dev};GFP* mice (Fig. 13C,D). When quantified, we found that NtsR1-GFP+ neurons outnumber NtsR2-GFP+ neurons approximately 18 to 1 (Fig. 13E). While the total number of NtsR1-GFP+ neurons in adult animals is about half of that observed in the developmental model, adult NtsR1-GFP+ neurons outnumber NtsR2-GFP+ neurons by 60 to 1 (Fig. 13F). Collectively, these data indicate that NtsR1 is the predominant isoform expressed on VTA neurons in development and adulthood, while NtsR2 is primarily

expressed on cells with glial morphology. Furthermore, our findings reveal that many cells express NtsR1 and NtsR2 at some stage of development, but in the adult brain, NtsR1 expression is confined to a subset of VTA cells.

NtsR1 is the Predominant Isoform on VTA DA Neurons: To define the neurochemical phenotype of NtsR1 and NtsR2 neurons, we examined VTA sections from the adult and developmental mouse models via immunofluorescence for tyrosine-hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis and a marker of DA neurons. This analysis revealed that nearly all (~98%) of VTA DA neurons express NtsR1 at some point in development (Fig. 14A, E), while about two thirds (~70%) of DA neurons actively co-express NtsR1 in adulthood (Fig. 14B, F). Interestingly, many TH-negative VTA neurons co-expressed NtsR1 in the developmental model (~30% of NtsR1-GFP+ neurons) but not the adult model (Fig. 14G, H). Thus during development NtsR1 is expressed in, and presumably regulates, both DA and other VTA neurons, but only mediates Nts actions in the adult brain via DA neurons. By contrast, the majority of the small population of NtsR2-GFP+ neurons in the developmental and adult models did not colocalize with TH (Fig. 14C, D, E, F) and NtsR2-GFP expression was found on only 6.5% of DA neurons in *NtsR2^{Dev};GFP* mice and <1% of DA neurons in *NtsR2^{Adult};GFP* mice (Fig. 14G, H). Taken together, these data support the hypothesis that NtsR1 is the dominant isoform regulating VTA DA neurons in both development and adulthood, and suggests that NtsR1 may also modulate non-DA neurons in the VTA during development.

Projections of VTA NtsR1 Neurons: Given that NtsR1 was expressed on some, but not all VTA DA neurons in adult mice, we investigated whether NtsR1 defined the subset of VTA DA neurons that project to the NA or the prefrontal cortex (PFC)[43], two major outputs of VTA DA neurons. To do this, we injected adult *NtsR1$^{\Delta NEO\text{-}Cre}$;GFP* mice in the VTA with the Cre-mediated tract tracer Ad-syn-mCherry[27] (Fig. 15A). In these brains, GFP identifies any cell that

expressed NtsR1 throughout lifespan, but only cells that actively express NtsR1 can undergo Cre-mediated recombination to express both GFP and the synaptophysin-mCherry fusion protein within cell bodies and terminals[27]. Thus, this method allows us to discriminate cells that only expressed NtsR1 during development from the adult cells that currently express NtsR1 and can mediate DA release. Visualization of the VTA revealed that nearly all mCherry+ neurons co-expressed GFP, however many GFP+ neurons within the injection site did not co-express mCherry (Fig. 15B, yellow arrows). These findings are consistent with our detection of almost twice as many NtsR1-GFP+ neurons in *NtsR1^{Dev};GFP* mice compared to *NtsR1^{Adult};GFP* mice (Fig. 12E, F) and confirms that NtsR1 expression, and hence induction of Cre, is confined to a limited set of VTA neurons in the adult brain. Out of 5 injected mice, 3 had mCherry-labeling confined to the VTA and were used for analysis (Fig. 15C). We also injected Ad-syn-mCherry into the VTA of *NtsR2$^{\Delta NEO\text{-}Cre}$;GFP* mice to verify the minor population NtsR2 neurons in the adult brain and define their projections. In contrast to the numerous mCherry-labeled neurons observed in *NtsR1$^{\Delta NEO\text{-}Cre}$;GFP* mice (Fig. 15D), examination of *NtsR2$^{\Delta NEO\text{-}Cre}$;GFP* mice revealed mCherry+ cells with predominantly stellate morphology in the VTA. This small population of VTA NtsR2 neurons provided <10 single terminals observed throughout the entire brain, including a minor projection to the NA and IPAC (Fig. 15 E, white arrows). These data confirm the dearth of NtsR2-expressing neurons in the adult VTA (Fig. 12F) and support a predominant role for NtsR1 in directly modifying VTA DA signaling.

Analysis of the projections from the well-targeted *NtsR1$^{\Delta NEO\text{-}Cre}$;GFP* mice is summarized in Fig. 16M. VTA NtsR1 neurons most densely project to sub-regions of the ventral striatum, including the NA core (NAc), NA shell (NAsh), and olfactory tubercle (OFT) (Fig. 16A, B, C). By contrast, the lack of terminals in the PFC implicates VTA NtsR1 neurons in regulating mesolimbic, but not mesocortical, DA signaling (Fig. 16D)[43]. We also observed dense VTA NtsR1 terminals within the interstitial nucleus of the posterior limb of the anterior commissure

(IPAC) and modest terminals in the neighboring stria terminals (ST) (Fig. 16F). The caudate putamen (CPu) contained sparse VTA NtsR1 terminals (Fig. 16E), consistent with the VTA providing less input to the dorsal striatum compared to SN[43,44]. A few mCherry-labeled terminals were observed in the ventral pallidum (VP) (Fig. 16G) and the central amygdala (CeA) (Fig. 16H), where DA release is associated with emotional learning and feeding[45-48]. While we detected few terminals within the lateral hypothalamus (LHA), the dense patch of mCherry+ fibers in the neighboring nigrostriatal tract likely represents VTA NtsR1 axons traversing the brain en route to the striatum (Fig. 16J, see nigrostriatal tract (ns)). Intriguingly, the lateral habenula (LHb) also contained many mCherry-labeled terminals (Fig. 16I), although VTA neurons projecting to the LHb do not release DA and instead inhibit the LHb to indirectly promote reward[49]. We also observed low to medium terminal density in the hindbrain, namely in the laterodorsal tegmental nucleus (LDTg) (Fig. 16K), the parabrachial nucleus (PBN) (Fig. 16L), and the dorsal raphe (DR) (Fig. 16M).

3.4 Discussion

Here, we used dual recombinase mice to identify NtsR1 and NtsR2 cells in the VTA to establish the precise cellular mediators of Nts action. We demonstrate that NtsR2 is predominately expressed on glial cells and only a small number of both DA and non-DAergic neurons throughout life. By contrast, NtsR1 is expressed on many VTA DA neurons during both development and adulthood. Furthermore, VTA NtsR1 neurons project to the ventral striatum, not the PFC, and hence are positioned to specifically modify mesolimbic DA signaling. Thus, our data are the first to demonstrate NtsR1 as the predominant receptor isoform by which Nts can directly engage DA neurons to modify DA-dependent signaling and physiology.

Our findings provide cellular resolution that will be essential to understand how Nts mechanistically acts in the VTA. Consistent with our finding of NtsR1 expression on some VTA

DA neurons, the application of Nts or Nts analogues into the VTA activates DA neurons, increases NA DA release, restrains food intake, and promotes reward behaviors, and these effects are reduced by NtsR1 antagonists or developmental deletion of NtsR1[1,25,26,28,29]. Similarly, Bose *et al.* suggested that Nts enhances excitatory input onto VTA DA neurons via NtsR1[50]. However, not all studies using pharmacological agents or receptor null mice have supported a predominant role for NtsR1 in directly modulating VTA DA signaling. For example, Kempadoo *et al.* found that low doses of intra-VTA Nts increased excitatory transmission to DA neurons via NtsR1, while high doses of Nts reduced excitatory input through an NtsR1-independent mechanism, implying involvement of NtsR2[29]. Similarly, Rouibi *et al.* reported that Nts action in the VTA reduces excitatory input to DA neurons via an NtsR1-independent mechanism, again suggesting involvement of NtsR2[28]. Our finding that <1% of adult VTA DA neurons co-express NtsR2 suggest it is unlikely that Nts directly modulates most DA neurons via NtsR2. One possible mechanism to resolve these discrepancies arises from our finding that NtsR2 is predominantly expressed on astrocytes, which might act via tripartite synapses to mediate local regulation of DA neurons[51]. Going forward, the ability to identify NtsR1 and NtsR2 cells will permit direct testing of how Nts engages these cells to orchestrate DA signaling.

Previously, NtsR expression was characterized using *in situ* hybridization (ISH), which labels mRNA but does not always provide sufficient signal to visualize morphologic features of the cell in which the transcript is expressed. An advantage of the dual recombinase strategy we used to identify NtsR-expressing cells is that Cre-induced GFP fills the entire cell, which allowed us to clearly distinguish between neuronal and glial morphologies. We were thus able to discern that most NtsR2 is expressed by glial cells throughout the brain, consistent with the diffuse, low intensity signal observed via ISH for *Ntsr2*[9,19,21,52]. Similarly, cultured astrocytes express NtsR2[20,41,42] and Nts modulates their activity[53,54]. Interestingly, because astrocytes also express endopeptidases that catabolize Nts[55-57], they might act as a sink to internalize and

degrade residual Nts at the synapse via NtsR2. Loss of NtsR2 signaling, as in NtsR2-null mice, could therefore lead to excess synaptic Nts and enhanced Nts-NtsR1 activation of VTA DA neurons, which may explain their increased striatal DA levels and hyperactivity[35].

VTA DA neurons are heterogeneous and have been defined by their projection targets (see [58,59] for review). We found that VTA NtsR1 neurons comprise ~70% of VTA DA neurons and project primarily to the NA, OFT, and IPAC, but not to other efferent targets of VTA NA neurons such as the PFC or hippocampus[43]. Given that VTA NtsR1 neurons are mesolimbic, they are likely to modulate the reinforcing properties of natural and pharmacologic rewards, which depend upon DA release to the NA[60-62 63,64]. Furthermore, our current data provides a cellular and circuit mechanism to explain how Nts signaling directly via VTA NtsR1 neurons can increase NA DA release and mediate conditioned reward[12,28,29,65-69]. The functional implications of the VTA NtsR1 projections to the IPAC are less clear. The IPAC is structurally related to the amygdala[70], and DA release in the amygdala has been implicated in emotional learning[46-48] and in regulation of food intake[45]. Finally, we noted several VTA NtsR1 projections to the LHb, a structure that mediates aversion. However, TH+ neurons that project to the LHb do not release DA, but rather inhibit the LHb, which then disinhibits VTA DA neurons[49]. Thus, the TH+ VTA projections to the LHb, including NtsR1-expressing neurons, may indirectly support reward rather than aversion. Overall, VTA NtsR1 neurons project to regions that can modify reward intake, suggesting that Nts action via the VTA may be necessary to drive pursuit of pharmacological and natural rewards, such as food.

A limitation of the developmental *NtsR1*$^{\Delta NEO\text{-}Cre}$*;GFP* and *NtsR2*$^{\Delta NEO\text{-}Cre}$*;GFP* models we developed is that GFP expression does not necessarily reflect cells with active NtsR1 or NtsR2 expression. Inducing Cre expression from the beginning of embryonic development in the developmental models leads to permanent GFP expression in all cells that expressed NtsR1 or

NtsR2 at any point in life. Comparing findings from developmental to adult models however, can inform how NtsR expression patterns change through life. For example, essentially all VTA DA neurons colocalize with GFP in *NtsR1^{Dev};GFP* mice whereas only ~70% colocalize in *NtsR1^{Adult};GFP* mice. These data imply that at some point in development ~30% of DA neurons transiently expressed NtsR1, but do not identify when they cease expressing NtsR1. Inducing FlpO expression at discrete time points across development will be required to define the temporal dynamics of NtsR1 expression. This could be accomplished by breeding mice to a tamoxifen-inducible FlpO deleter line, whereby injection of tamoxifen would induce Cre expression at any desired point in development[71] to label cells actively expressing NtsR1.

While NtsR1 is robustly expressed on VTA DA neurons throughout life, our *NtsR1$^{NEO\text{-}Cre}$;GFP* models do not distinguish between neurons that express NtsR1 on the soma/dendrites vs. synaptic terminals. The subcellular localization of NtsR1 is functionally important because Nts-NtsR1 signaling elicits different behavioral effects via either pre- or post-synaptic mechanisms. Nts-NtsR1 signaling on DA cell bodies in the VTA increases DA release and locomotor activity similar to psychostimulants[12,65-67,72,73]. By contrast, Nts-NtsR1 action in the NA suppresses locomotor activity induced by AMPH, cocaine, and DA itself, similar to antipsychotic medications[74-76], and is thought to be mediated by NtsR1 expressed pre-synaptically on DA terminals in the striatum. Studies using immunohistochemistry and autoradiography suggest that NtsR1 binding sites are present on DA terminals in the ventral and dorsal striatum[77-79]. Furthermore, radiolabeled Nts injected in to the striatum accumulates in the cell bodies of VTA DA neurons[80-82], indicating that Nts binds DA neurons at presynaptic terminals and is internalized. Collectively, VTA Nts-NtsR1 signaling appears to have opposing behavioral outcomes depending on whether it occurs pre- or post-synaptically, and this should be taken into account when interpreting future data from *NtsR1$^{NEO\text{-}Cre}$* mice.

Our work indicates vast expression of NtsR1 throughout the brain in *NtsR1^{Dev};GFP* mice, and a heretofore unappreciated potential role for Nts to mediate development of the central nervous system. Within the VTA, *NtsR1^{Dev};GFP* mice showed nearly 100% of VTA DA neurons colocalizing with GFP, which represented ~65% of all GFP+ neurons. Curiously, this is similar to the overall distribution of DA and non-DA neurons in adults (~65% TH+, 35% TH-)[58] and suggests that at some point in life, almost every VTA neuron, both DA and non-DAergic, expresses NtsR1. Nts-NtsR1 signaling may thus play a critical role in establishing VTA circuits during development. Mice null for NtsR1 may therefore suffer from abnormal formation of the VTA DA system that causes aberrant DA signaling, locomotor activity, body weight, anxiety, sleep, and exaggerated response to psychostimulants that has been reported to occur in this line[1,2,27,35,36]. Our data also identify a substantial population of VTA NtsR1 neurons that persist in the adult brain, and hence signify roles for Nts signaling via NtsR1 in regulating normal physiology. Going forward, using *NtsR1$^{NEO\text{-}Cre}$* mice will permit selective manipulation of the VTA NtsR1 neurons in the adult brain to discern how they contribute to DA signaling, physiology and behavior.

3.5 Methods

Generation of NtsR1$^{NEO\text{-}Cre}$ and NtsR2$^{NEO\text{-}Cre}$ Knock-In Mice: NtsR1$^{NEO\text{-}Cre}$ and *NtsR2$^{NEO\text{-}Cre}$* targeting vectors were generated by inserting an IRES-Cre between the stop codon and the polyadenylation site of the sequence encoding the 3' end of the mouse *Ntsr1* gene, with an *frt*-flanked NEO cassette placed upstream of the IRES-Cre. The linearized targeting vector was electroporated into mouse R1 embryonic stem (ES) cells (129sv background) and cells were selected with G418. DNA from ES cell clones was analyzed via qPCR for loss of homozygosity using Taqman primer and probes for the genomic *Ntsr1* or *Ntsr2* insertion sites (*NtsR1:* Forward: TCTGATGTTGGACTTGGGTTC, Reverse: TCTGATGTTGGACTTGGGTTC, Probe: TCTGATGTTGGACTTGGGTTC. *NtsR2:* Forward:

ACCCATCAGATAAGCCATGC, Reverse: GTGGGAAGTTGAGGGCAG, Probe: GTCTAAGCGGACCTACTGACCCA). *NGF* was used as a copy number control[83]. Putative positive ES clones were expanded, confirmed for homologous recombination by Southern blot and injected into mouse C57BL/6 blastocysts to generate chimeras. Chimeric males were mated with C57BL/6 females (Jackson Laboratory), and germline transmission was determined initially via progeny coat color, then confirmed via conventional PCR for IRES-Cre.

Breeding and Genotyping: Mice were bred and housed in a 12h light/12h dark cycle with *ad libitum* access to water and food (Harlan Teklad #7913). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Heterozygous *NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice were bred to either FlpO deleter mice (Jax Stock #012930), a Cre-inducible Rosa$^{eGFP\text{-}L10a}$ reporter[84], or C57/Bl6 wild-type mice to maintain the lines. *Developmental Model: NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice were bred to a FlpO deleter line and progeny that inherited the FlpO allele (*NtsR1*$^{\Delta NEO\text{-}Cre}$ and *NtsR2*$^{\Delta NEO\text{-}Cre}$) were subsequently mated to a Cre-inducible *Rosa*$^{eGFP\text{-}L10a}$ reporter, generating *NtsR1*^{Dev}*;GFP* and *NtsR2*^{Dev}*;GFP* mice. Progeny that were heterozygous for both *IRES-Cre* and *GFP* alleles were used for analysis. *Adult Model: NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice were mated directly to Cre-inducible *Rosa*$^{eGFP\text{-}L10a}$ animals, producing heterozygous *NtsR1*$^{NEO\text{-}Cre}$*;GFP* and *NtsR2*$^{NEO\text{-}Cre}$*;GFP* progeny. These animals were then injected with FlpO adenovirus in adulthood (see below) to generate *NtsR1*^{Adult}*;GFP* and *NtsR2*^{Adult}*;GFP* study mice. All animals were genotyped by standard PCR using the following primer sequences: *IRES-Cre*: Forward: GGACGTGGTTTTCCTTTGAA. Reverse: AGGCAAATTTTGGTGTACGG. *Rosa26*$^{EGFP\text{-}L10a}$: Mutant forward: TCTACAAATGTGGTAGATCCAGGC, WT forward: GAGGGGAGTGTTGCAATACC, Common: CAGATGACTACCTATCCTCCC. *FlpO*: Mutant: GCGAAGAGTTTGTCCTCAACC, Common: GCG AAG AGT TTG TCC TCA ACC, Wild-type: GGAGCGGGAGAAATGGATATG. Adult male and female mice of each model were studied.

Surgery: Adult *NtsR1^{Adult};GFP* and *NtsR2^{Adult};GFP* mice received a pre-surgical injection of carprofen (5mg/kg *s.c.*) and were anesthetized with 3-4% isoflurane/O_2 in an induction chamber before being placed in a stereotaxic frame (Kopf). Under 1-2% isoflurane, access holes were drilled in the skull allowing a guide cannula with stylet (PlasticsOne) to be lowered into the lateral ventricles (A/P: -0.22, M/L: +/- 1.0, D/V: -2.0). Mice were bilaterally injected with 1µL FlpO adenovirus (Vector Biolabs), which was infused at a rate of 1µL/minute. The animals recovered for 10 days prior to perfusion to permit sufficient time for FlpO-mediated excision of the *frt*-flanked NEO cassette and GFP expression. For tracing studies, *NtsR1$^{\Delta NEO\text{-}Cre}$;GFP* mice were injected unilaterally in the VTA (A/P: -3.2, M/L: +/-0.48, D/V: -4.65) with 75-100nL of Ad-syn-mCherry, an adenovirus expressing a Cre-dependent synaptophysin-mCherry fusion protein[27] (provided by Martin Myers, University of Michigan). Mice recovered for 7-10 days after surgery to allow for Cre-mediated recombination and synaptophysin-mCherry expression at pre-synaptic terminals.

Perfusion and Immunofluorescence: Mice were treated with a lethal dose of *i.p.* pentobarbital followed by transcardial perfusion with 10% neutral-buffered formalin (Fisher Scientific). Brains were removed, post-fixed in 10% formalin overnight at 4˚C, dehydrated with 30% sucrose in PBS for 2-3 days, and sectioned into 30 µm slices using a sliding microtome (Leica). Brain sections were then analyzed by immunofluorescence or immunohistochemistry as previously described[27,37]. For characterization of NtsR1 and NtsR2 expression, sections were exposed to chicken anti-GFP (1:2000, Abcam) and mouse anti-TH (1:1000, Millipore), followed by incubation with species-specific secondary antibodies conjugated to AlexaFluor 488 or 568 fluorophores (Life Technologies or Jackson ImmunoResearch). Brains were analyzed using an Olympus BX53 fluorescence microscope outfitted with FITC and Texas Red filters. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe). Five representative

sections spanning the entire VTA were selected from each mouse and were used to determine the number and colocalization of GFP and/or TH positive neurons.

Statistics. All data were analyzed in Prism 6 (GraphPad) using unpaired t-tests. Bar graphs represent mean ± SEM.

APPENDIX

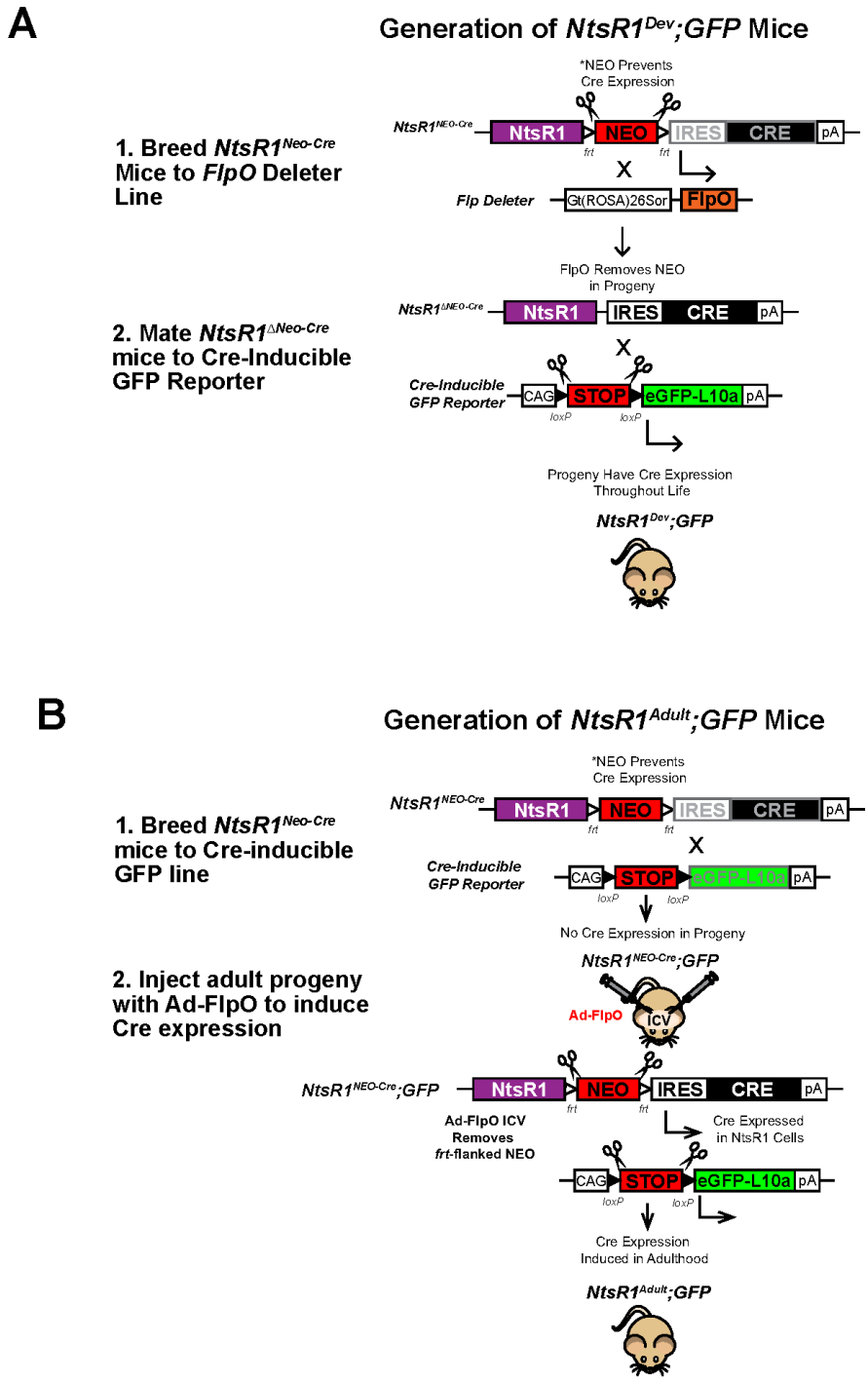

Figure 12. Generation of mouse models to identify developmental vs. adult expression patterns of NtsR1 and NtsR2.

Figure 12 (*cont'd*). A) Schematic demonstrating how constitutive Cre expression is induced in *NtsR1*$^{Neo\text{-}Cre}$ mice, resulting in GFP labeling of any neuron that expresses NtsR1 throughout the lifespan (*NtsR1*^{Dev} model). **B)** Schematic depiction of how Cre expression is suppressed until adulthood in *NtsR1*$^{Neo\text{-}Cre}$ mice, resulting in GFP labeling restricted to cells actively expressing NtsR1 in adult animals (*NtsR1*^{Adult} model). **The same strategies depicted in **A-B** were used in NtsR2*$^{Neo\text{-}Cre}$ *mice.*

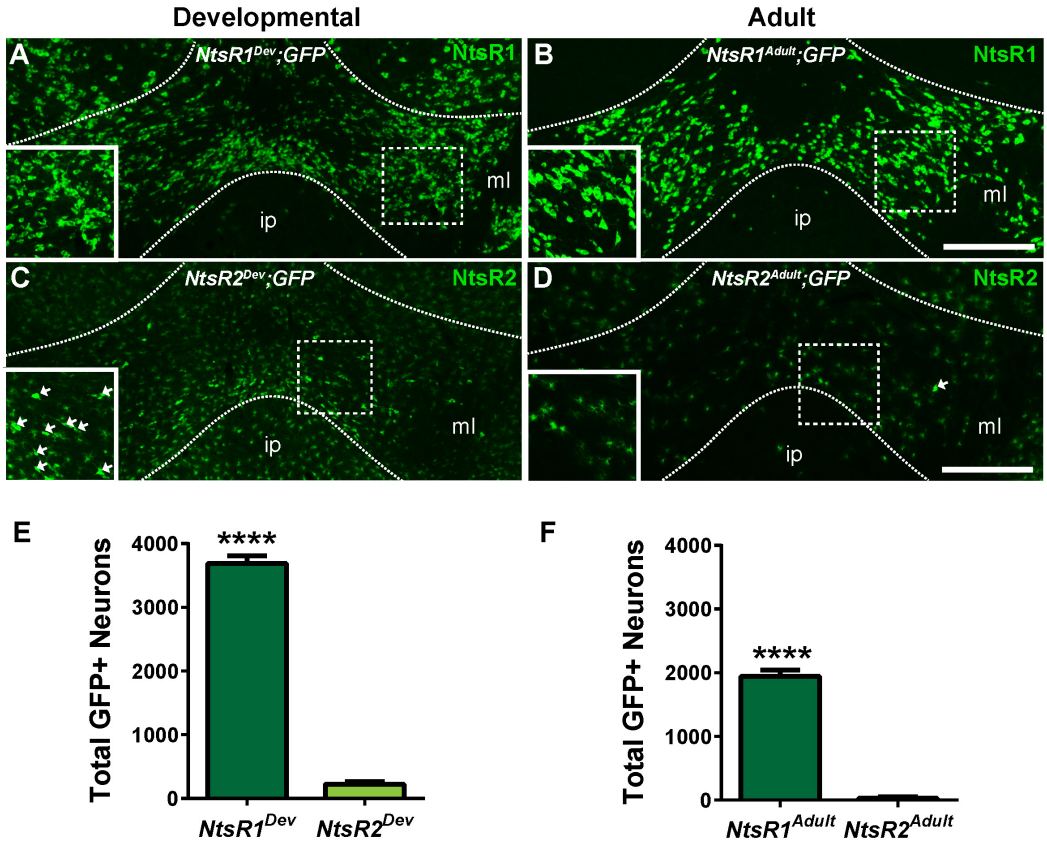

Figure 13. Developmental vs. adult expression of NtsR1 and NtsR2 in the VTA. *NtsR1*$^{NEO\text{-}Cre}$ and *NtsR2*$^{NEO\text{-}Cre}$ mice were bred to a Cre-inducible GFP reporter line and Cre expression was induced constitutively from conception (developmental expression) or only in adulthood. **A-B)** Developmental and adult distributions of NtsR1 neurons. **C-D)** Developmental and adult distributions of NtsR2 neurons. GFP+ cells have either neuronal (white arrows) or glial morphology. *ip=interpeduncular nucleus, ml=medial lemniscus*. Scale bars represent 200 µm. Insets are digital enlargements of the areas within dashed boxes. **E, F)** Total number of GFP+ NtsR1 or NtsR2 neurons in developmental and adult models in the VTA (*NtsR1*^{Dev} n=3, *NtsR2*^{Dev} n=3, *NtsR1*^{Adult} n=3, *NtsR2*^{Adult} n=6). Each bar represents mean ± SEM and data were analyzed by unpaired t-tests.

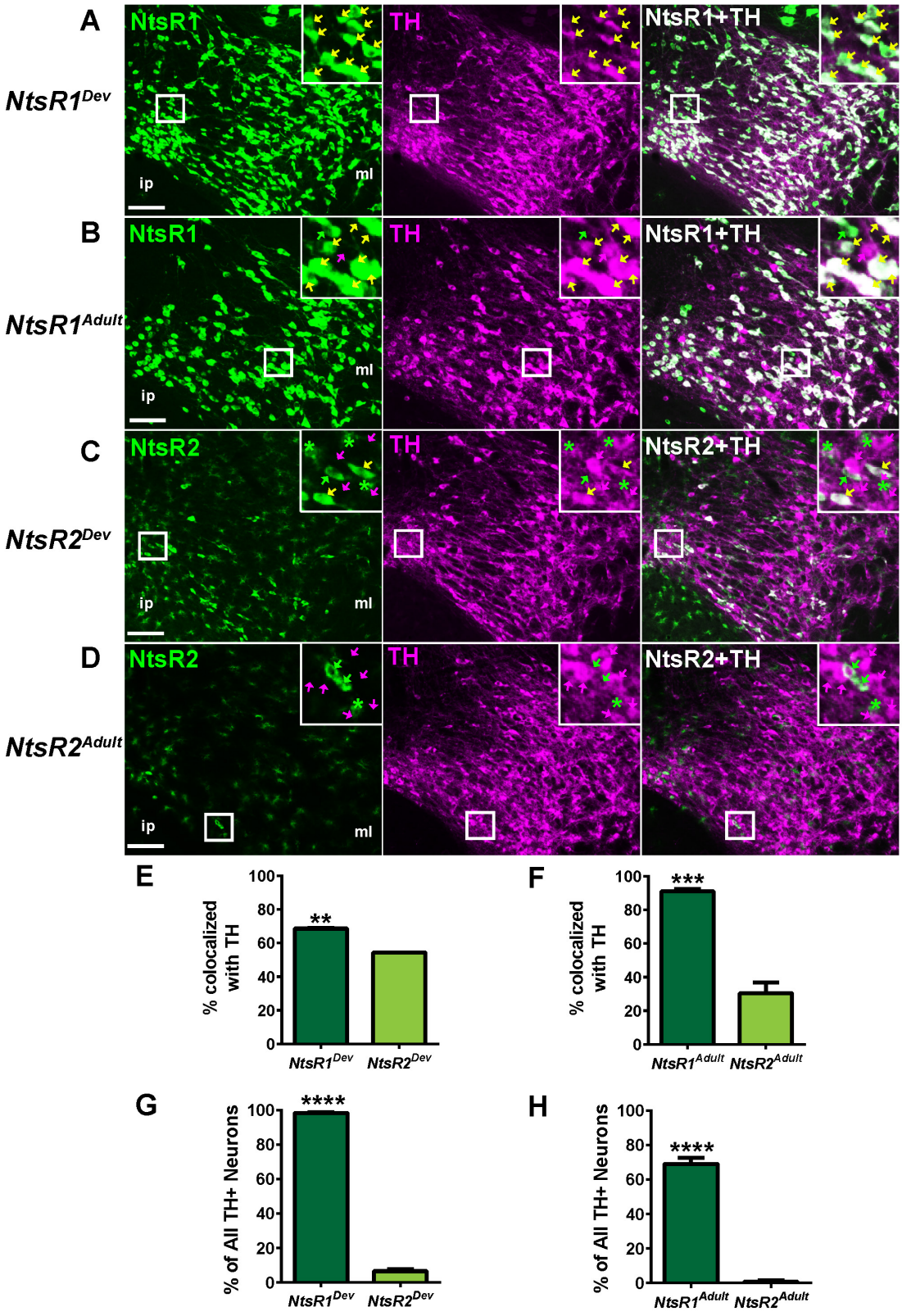

Figure 14. NtsR1 and NtsR2 colocalization with TH in development compared to adulthood.

Figure 14 (*cont'd*). Representative images showing TH co-expression in the VTA with **A)** developmental NtsR1-GFP+ neurons, **B)** adult NtsR1-GFP+ neurons, **C)** developmental NtsR2-GFP+ neurons **D)** adult NtsR2-GFP+ neurons. Yellow arrows = TH/GFP+ colocalized neurons, green arrows = GFP+ only neurons, pink arrows = TH+ only neurons, green asterisks = GFP+ glia. Scale bars=100μm. *ml=medial lemniscus, ip=interpeduncular nucleus*. Insets are digital enlargements of the indicated boxed areas. Percentage of NtsR1 and NtsR2 neurons that colocalize with TH in **E)** developmental and **F)** adult expression models. Percentage of all VTA TH+ neurons that colocalize with NtsR1 or NtsR2 in **G)** developmental and **H)** adult models. (*NtsR1*^{Dev} n=3; *NtsR2*^{Dev} n=3; *NtsR1*^{Adult} n=3; *NtsR2*^{Adult} n=6). Bars represent mean ± SEM and data were analyzed by unpaired t-tests.

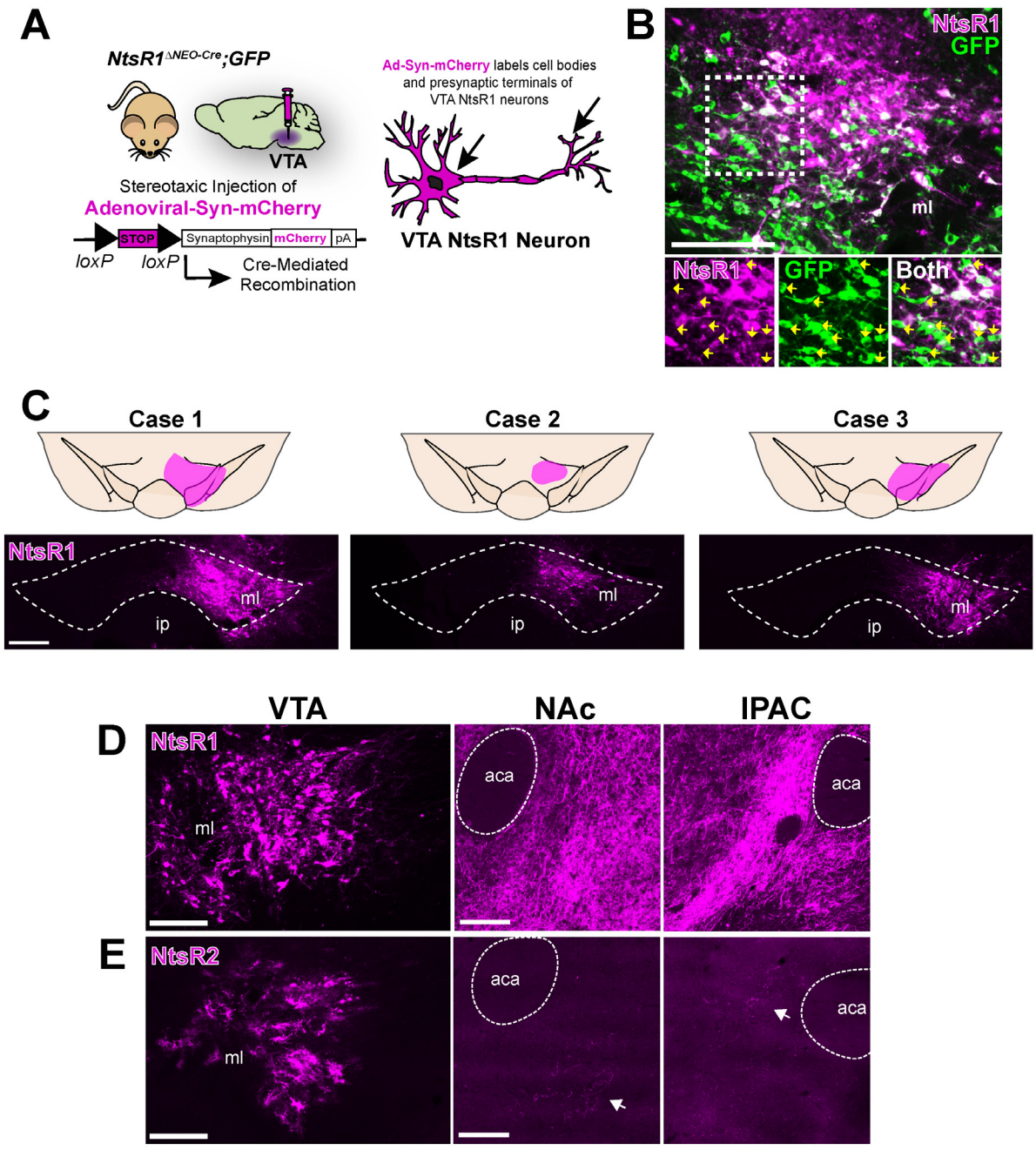

Figure 15. Ad-syn-mCherry reveals projections of VTA NtsR1 neurons. A) Schematic showing *NtsR1*$^{\Delta NEO\text{-}Cre}$*;GFP* mice injected in the VTA with Ad-syn-mCherry, which labels VTA NtsR1 projections by expression of a Cre-inducible synaptophysin-mCherry fusion protein. **B)** VTA of *NtsR1*$^{\Delta NEO\text{-}Cre}$*;GFP* mouse injected with Ad-syn-mCherry showing GFP+ neurons that do not co-express mCherry (yellow arrows), representing neurons that transiently expressed NtsR1 during development. **C)** Distribution of mCherry+ cell bodies within the VTA from 3 individual *NtsR1*$^{\Delta NEO\text{-}Cre}$*;GFP* mice, confirming selective labeling of VTA NtsR1 neurons. Comparison of VTA injection site and mCherry labeled terminals in the NAc and IPAC of **D)** *NtsR1*$^{\Delta NEO\text{-}Cre}$*;GFP* mice vs. **E)** *NtsR2*$^{\Delta NEO\text{-}Cre}$*;GFP* mice. Scale bars represent 100µm. *ml=medial lemniscus, ip=interpeduncular nucleus, aca=anterior commissure*.

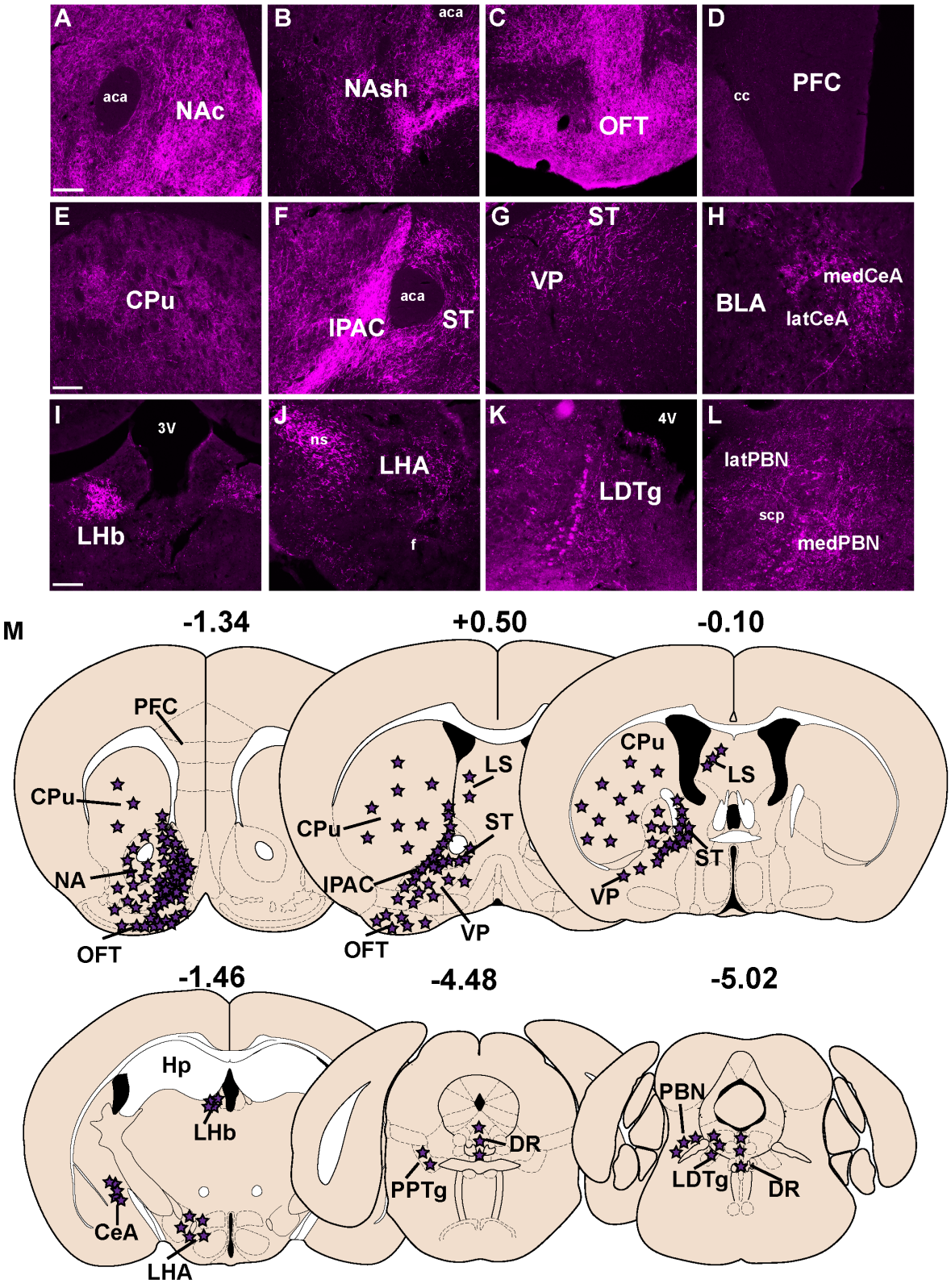

Figure 16. Projections of VTA NtsR1 neurons.

Figure 16 (*cont'd*). Ad-syn-mCherry-labeled terminals in the **A)** nucleus accumbens core (NAc), **B)** nucleus accumbens shell (NAsh), **C)** olfactory tubercle (OFT), **D)** absence of terminals in the prefrontal cortex (PFC), **E)** caudate/putamen (CPu), **F)** lPAC and stria terminals (ST), **G)** ventral pallidum (VP), **H)** central amygdala (CeA), **I)** lateral habenula (LHb), **J)** lateral hypothalamic area (LHA), **K)** laterodorsal tegmentum (LDTg), **L)** parabrachial nucleus (PBN). **M)** Schematic summarizing projections of VTA NtsR1 neurons (purple stars). Scale bars represent 100μm. *aca=anterior commissure, cc=corpus callous, BLA=basolateral amygdala, 3V= third ventricle, ns= nigrostriatal tract, f=fornix, 4V=fourth ventricle, scp=superior cerebellar peduncle*.

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Chapter 4. Neurotensin Receptor-1 Identifies a Subset of Ventral Tegmental Dopamine Neurons that Coordinate Energy Balance

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This chapter is a modified version of a manuscript accepted to Cell Reports.

4.1 Abstract

Dopamine (DA) neurons in the ventral tegmental area (VTA) are heterogeneous and differentially direct ingestive and locomotor behaviors that impact energy balance. Identification of which VTA DA neurons mediate behaviors that limit weight gain has been hindered, however, by the lack of molecular markers to distinguish VTA DA populations. Here, we identified a specific subset of VTA DA neurons that express neurotensin receptor-1 (NtsR1) and project to the nucleus accumbens (NA). Genetically targeted ablation of VTA NtsR1 neurons uncouples motivated feeding and physical activity, biasing behavior toward energy expenditure and protecting mice from age-related and diet-induced weight gain. VTA NtsR1 neurons thus represent the first molecularly defined subset of mesolimbic DA neurons that are essential for the coordination of energy balance. Modulation of VTA NtsR1 neurons may therefore be useful to promote behaviors that prevent the development of obesity.

4.2 Introduction

Obesity is caused by overconsumption of calorie-dense food combined with insufficient physical activity, and predisposes individuals to myriad chronic conditions that shorten life span. Proper diet and exercise are important for maintaining healthy weight but these lifestyle modifications are difficult to maintain long-term. Consequently, most individuals that lose weight

through changes in diet and activity tend to regain it over time[1]. Therapeutic strategies to suppress feeding and encourage physical activity would therefore be helpful to prevent weight gain and combat the obesity epidemic.

The motivated feeding and locomotor behaviors that influence body weight are regulated, in part, by dopamine (DA) neurons in the ventral tegmental area (VTA) that release DA within the nucleus accumbens (NA) and prefrontal cortex (PFC)[2,3]. DA itself is essential for energy balance, demonstrated by the aphagia, hypolocomotion, reduced body weight and early lethality of mice that genetically lack DA[4]. Disruptions in DA signaling are also observed in obese rodents and humans, suggesting that inappropriate regulation of DA circuits contributes to weight gain[5-7]. Yet, the mechanisms by which VTA DA neurons orchestrate energy balance remain poorly understood. One emerging theory is that VTA DA neurons are not homogenous, but in fact consist of subsets of neurons that coordinate distinct aspects of feeding and locomotor activity. Indeed, VTA DA neurons can be differentiated according to their anatomical inputs and projections[8,9] or their electrophysiological firing properties[10]. VTA DA neurons projecting to the NA are primarily activated by “rewarding” stimuli, whereas DA neurons projecting to the PFC are regulated by “aversive” cues[8]. VTA DA neurons may also contain the classical neurotransmitters GABA or glutamate[11,12], whose co-release with DA at distinct projection sites may either promote or suppress feeding[13]. Collectively, these data suggest the interesting, and as yet untested possibility that distinct VTA DA circuits promote or suppress obesogenic behaviors. Unfortunately, neither projection output nor transmitter content identifies which specific VTA DA neurons may be leveraged to treat energy balance disorders. Certainly, molecular markers to differentiate functionally distinct subsets of VTA DA neurons would be useful in this regard, but to date no such markers have been identified.

Neuropeptides such as orexin, corticotropin releasing factor (CRF), glucagon-like peptide-1 (GLP-1), and neurotensin (Nts) differentially modify DA-dependent behaviors[14-17], thus we reasoned that VTA DA neurons might be distinguished by their expression of neuropeptide receptors. Of these systems, Nts holds particular promise for modifying obesogenic behaviors, since pharmacologic administration of Nts in the VTA restrains feeding, increases locomotor activity and induces weight loss in obese rodents[18-20]. Nts signals via neurotensin receptors -1 and -2 (NtsR1 and NtsR2) that are expressed in the VTA[21,22], and Nts enhances the activation of VTA DA neurons and DA release in the NA[14,23]. The lack of reagents to identify cells expressing NtsRs, however, has hampered a more detailed understanding of how Nts mechanistically engages DA neurons. To overcome this challenge, we used Cre-lox technology to selectively visualize and manipulate NtsR1 or NtsR2-expressing cells in mice and in doing so identified a unique subset of VTA DA neurons that express NtsR1. Furthermore, we found that disrupting VTA NtsR1 neurons alters DA-mediated behaviors to prevent weight gain, suggesting that this subset of VTA neurons may represent a novel cellular target for preventing the development of obesity.

4.3 Results

NtsR1 and NtsR2 Identify Distinct VTA Cell Types: We crossed existing transgenic *NtsR1*^{Cre} mice and newly generated knock-in *NtsR2*^{Cre} mice onto a Cre-inducible-GFP reporter line, such that progeny expressed GFP selectively in NtsR1 or -2 expressing cells, respectively (Fig. 17). These models revealed striking differences in the morphology, number, and distribution of NtsR1-GFP and NtsR2-GFP cells throughout the rostrocaudal axis of the VTA (Fig. 18A). While a few NtsR2-GFP cells appeared to be neurons (Fig. 18A, white arrows), the vast majority were stellate-shaped cells consistent with glial morphology (Fig. 18A, yellow arrows). By contrast, NtsR1-GFP cells were exclusively neuronal (Fig. 18A) and more numerous than NtsR2-GFP neurons. The NtsR1-GFP neurons were predominantly located in

the rostral VTA (bregma-3.40 to -3.05), while the few NtsR2-GFP neurons clustered along the ventral-medial border of the caudal VTA (bregma -3.64), a region enriched in GABA-ergic neurons[24].

Given the distinct morphology and distribution of NtsR1-GFP and NtsR2-GFP cells, we next used immunolabeling to determine if they represent distinct cellular populations. The NtsR2-GFP cells with stellate morphology co-localized with the astrocyte marker S100, confirming that most NtsR2 cells are glia (Fig. 18B, yellow arrows). By contrast, none of the NtsR1-GFP cells or the few NtsR2-GFP cells with neuronal morphology co-localized with S100 (Fig. 18B, C, white arrows). Instead >90% of NtsR1-GFP neurons co-expressed tyrosine hydroxylase (TH), a marker of DA neurons (Fig. 18E-I), consistent with our previous findings[25]. Only ~30% of the much smaller population of NtsR2-GFP neurons co-expressed TH, representing ≤1% of all VTA DAergic neurons (Fig. 18D, F-I). Collectively, these data reveal that NtsR1 and NtsR2 are expressed in non-overlapping VTA subpopulations, that NtsR1 is the predominant receptor isoform expressed on VTA neurons, and that VTA NtsR1 neurons are a subset of all VTA DA neurons.

VTA NtsR1 Neurons are a Projection-Defined Subset of VTA DA Neurons: We next investigated whether the novel subset of VTA NtsR1 neurons are a projection-specific population of VTA DA neurons by injecting the Cre-mediated anterograde tract tracer Ad-Syn-mCherry[25] into the VTA of transgenic *NtsR1*^{Cre} mice (Fig. 19A, B). This method revealed that VTA NtsR1 neurons densely innervate the ventral striatum, particularly the NA core and shell (NAc and NAs) and the nearby olfactory tubercle (OFT) (Fig. 19C, E). The second most densely innervated structure was the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), which adjoins the NA (Fig. 19C, E). Moderate terminal density was observed in the ventral caudate/putamen (CPu) just adjacent to the NA (Fig. 19C, E), as well as

sparse projections within the caudal CPu and basolateral amygdala (BLA). By contrast, no projections were found in the central amygdala (CeA), hippocampus (Hipp) or the PFC (Fig. 19C-E). Thus, NtsR1 defines a subset of VTA DA neurons that regulate the NA and contiguous subregions, and can be used as a molecular marker to parse mesolimbic vs. mesocortical populations of VTA DA neurons.

Loss of VTA NtsR1 Neurons Alters Energy Balance to Promote Leanness: To determine if VTA NtsR1 neurons contribute to the control of energy balance, we specifically ablated VTA NtsR1 neurons in adult mice (Fig. 20A). Adult *NtsR1^{Cre};GFP* mice received bilateral injections of an adeno-associated virus (AAV) expressing Cre-dependent Diphtheria Toxin A subunit (AAV-DTA) into the VTA, and DTA expression selectively kills VTA NtsR1 neurons (*NtsR1^{DTA}* mice). Surrounding cells remain intact since DTA is not transmitted outside of Cre-expressing neurons[26]. Separate *NtsR1^{Cre};GFP* mice received bilateral VTA injections of AAV-GFP and retained intact NtsR1 neurons (*NtsR1^{GFP}* controls). We verified that AAV-DTA sufficiently depleted VTA NtsR1 neurons as early as two weeks post-surgery, but the remaining TH+ DA neurons confirmed that this method does not ablate DA neurons that do not express NtsR1 (Fig. 20B). We therefore compared *NtsR1^{DTA}* mice and controls to reveal the role of VTA NtsR1 neurons in energy balance. *NtsR1^{DTA}* mice exhibited significantly reduced body weight compared to *NtsR1^{GFP}* controls by 4 wk post-surgery and remained leaner (Fig. 20C) with decreased body fat percentage (Fig. 20D). Surprisingly, *NtsR1^{DTA}* mice consumed increasingly more chow (Fig. 20E) and drank more water over the study compared to *NtsR1^{GFP}* controls (Fig. 21A), suggesting that loss of VTA NtsR1 neurons promotes ingestive behavior. While the increased energy intake of *NtsR1^{DTA}* mice was surprising given their decreased body weight (Fig. 20G, I), we wondered if augmented energy expenditure might explain why these mice remain lean. To this end, we noted hyperactivity of *NtsR1^{DTA}* mice in their home cages and metabolic analysis confirmed increased locomotor activity compared to *NtsR1^{GFP}* controls (Fig.

20F). Further, when energy intake was plotted against energy expenditure and the distributions were tested for differences using linear regression, we found a significantly increased y-intercept in the *NtsR1*^{DTA} group at 4 but not 16 weeks (Fig. 20S, T). This indicates that at a given caloric intake, *NtsR1*^{DTA} mice use more energy than controls and thus may not be able to appropriately adjust caloric intake to support their elevated activity at least 4 weeks after ablation, which likely contributes to their reduced body weight and adiposity. Lack of differences between groups at 16 weeks (Fig. 20T) suggests that compensatory feeding eventually occurs with time, but is not sufficient to match the normal body weight and adiposity levels of controls (Fig. 20C, D).

Increased locomotion generally demands a commensurate increase in respiration to support the activity, and indeed the hyperlocomotive *NtsR1*^{DTA} mice had increased VO_2 and VCO_2 (Fig. 21C-G) and exhibited increasingly higher rates of energy expenditure (adjusted for weight) over the course of the study compared to controls (Fig. 20H, J). The respiratory exchange ratio (RER) was unaffected (Fig. 21E-H) indicating that loss of VTA NtsR1 neurons does not alter energy substrate usage. Collectively, these data indicate that loss of VTA NtsR1 neurons biases energy balance toward increased physical activity and energy expenditure to promote leanness.

Given the lean phenotype of mice lacking VTA NtsR1 on a normal chow diet, we hypothesized that they would be protected from diet-induced obesity. *NtsR1*^{DTA} mice gained significantly less weight on high fat (HF) diet and were completely protected from diet-induced increase in body fat percentage compared to control mice (Fig. 20K, L). Although *NtsR1*^{DTA} mice consumed more HF diet (Fig. 20M) and had higher weight-adjusted energy intake than *NtsR1*^{GFP} controls (Fig. 20O, Q), they also exhibited escalating increases in locomotor activity (Fig. 20N), VO_2, VCO_2 (Fig. 21I-N) and energy expenditure (Fig. 20P, R) over the study. Thus,

NtsR1^{DTA} mice remain lean in spite of their obesogenic diet, and this was due to chronically increased locomotor activity and energy expenditure.

Loss of VTA NtsR1 Neurons Alters Hedonic and Motivated Sucrose Intake: Loss of VTA NtsR1 neurons could also promote leanness by reducing the hedonic value of food, leading to insufficient caloric intake needed to balance increased energy expenditure. Surprisingly, *NtsR1*^{DTA} mice prefer and overconsume sucrose compared to control mice (Fig. 22A-C). We reasoned that if *NtsR1*^{DTA} mice drink more sucrose to meet homeostatic need then their intake should be suppressed by treatment with the appetite-suppressing hormone, leptin[27]. While leptin mildly diminished sucrose preference in *NtsR1*^{GFP} controls, it did not suppress the sucrose preference of *NtsR1*^{DTA} mice (Fig. 22D). These data suggest that loss of VTA NtsR1 neurons prevents appropriate response to leptin, compromising the ability to adapt caloric intake and locomotor behaviors as needed to maintain normal weight.

We next examined whether loss of VTA NtsR1 neurons might have uncoupled motivated feeding required to offset increased energy expenditure. Despite being hyperactive, *NtsR1*^{DTA} mice correctly learned to self-administer sucrose in a time frame similar to that of control mice (Fig. 22E, H), confirming that loss of VTA NtsR1 neurons does not compromise learning or attention processes required for motivated intake. Furthermore, *NtsR1*^{DTA} and *NtsR1*^{GFP} work equally to obtain sucrose (Fig. 22I, J), thus loss of VTA NtsR1 neurons does not prevent sucrose “wanting”. Given the extreme energy demands of *NtsR1*^{DTA} mice, however, their lack of increased motivated responding is counterintuitive: these mice should be more motivated than controls to consume calories[28]. We therefore reasoned that VTA NtsR1 neurons may be required to coordinate signals of energy status with appropriate adaptations in motivated behavior needed to maintain body weight. If this were true, then *NtsR1*^{DTA} mice lacking VTA NtsR1 neurons should be unable to adjust their motivated intake in response to peripheral

energy cues. As expected, *NtsR1*^{GFP} control mice were less motivated to nose-poke for sucrose in response to cues of energy sufficiency such as sucrose pre-feeding[29] or leptin[30], but *NtsR1*^{DTA} mice did not adjust their intake in response to these cues (Fig. 22K, L). Taken together, these findings reveal an important role for VTA NtsR1 neurons in linking metabolic status and motivated feeding necessary to maintain body weight.

Loss of VTA NtsR1 Neurons Modifies DA-Mediated Locomotor Activity: The hyperactivity of *NtsR1*^{DTA} mice suggested that they retained DA-mediated locomotor behavior. Consistent with this, open field locomotor activity of *NtsR1*^{DTA} and *NtsR1*^{GFP} mice was not disrupted by PBS-injection stress (Fig. 23A, B, Test A), whereas treatment with the D1 receptor antagonist SCH23390 (0.1mg/kg *i.p.*) blunted locomotor activity in both groups (Fig. 23A, B, Test B). We therefore used amphetamine (AMPH)-induced locomotor activity to assess the integrity of the mesolimbic DA system in *NtsR1*^{DTA} mice. As expected, AMPH treatment (4mg/kg *i.p.*) significantly increased locomotor activity in *NtsR1*^{GFP} mice, but *NtsR1*^{DTA} mice did not respond with increased activity (Fig 23D-G). Instead, we anecdotally observed that AMPH-treated *NtsR1*^{DTA} mice engaged in stationary, stereotypic movements typical with ceiling effects of DA signaling, which account for their apparent decrease in ambulatory activity (Fig. 23D-G). Since *NtsR1*^{DTA} mice lack a subset of VTA DA neurons that projects to the NA, we examined the ability of AMPH to induce DA release in the NA using AMPH-induced cFos immunoreactivity to identify activated NA neurons. AMPH treatment significantly increased numbers of cFos-positive cells in the NA of both *NtsR1*^{DTA} and control mice, although the response was non-significantly blunted in *NtsR1*^{DTA} mice, consistent with loss of some, but not all NA-projecting VTA DA neurons (Fig. 23H, I). Together, these data indicate that loss of VTA NtsR1 neurons does not completely abolish mesolimbic signaling. In fact, the high baseline locomotor activity and AMPH-induced stereotypy observed in *NtsR1*^{DTA} mice may indicate enhanced DA signaling, similar to compensatory increases in extracellular DA that occur with partial loss of DA

neurons[31]. Although hyperactivity and excessive DA have been linked with anxiety, there were no differences in anxiety-like behavior between *NtsR1*^{GFP} and *NtsR1*^{DTA} mice as assessed via open field center activity (Fig. 23C, F) and elevated plus maze (EPM) (Fig. 24).

Loss of VTA NtsR1 Neurons Modifies the Mesolimbic DA System: Lastly, we examined how loss of VTA NtsR1 neurons impacts the integrity of the mesolimbic DA system. *NtsR1*^{DTA} mice have reduced TH and DAT immune-labeled terminals in the NA and OFT compared to control mice (Fig. 25A, B) but residual immunoreactivity suggests preservation of non-NtsR1 expressing mesolimbic neurons. To assess molecular alterations in the VTA, we administered unilateral VTA injections of AAV-DTA or AAV-GFP to *NtsR1*^{Cre}*;GFP* mice and analyzed the fold difference in gene expression between the injected and un-injected sides; we refer to these as *NtsR1*$^{GFP\text{-}Uni}$ and *NtsR1*$^{DTA\text{-}Uni}$ mice (Fig. 25C). As expected, *NtsR1* and DA-associated transcripts such as *Th*, *Dat* and *D2* were significantly reduced in the VTA of *NtsR1*$^{DTA\text{-}Uni}$ mice, consistent with the loss of DAergic VTA NtsR1 cell bodies (Fig. 25D). The slight reduction in *vglut2* and *vgat* in *NtsR1*$^{DTA\text{-}Uni}$ mice suggests that VTA NtsR1 neurons may also contain glutamate and/or GABA in addition to DA. *Ntsr2* expression, however, did not differ between *NtsR1*$^{DTA\text{-}Uni}$ mice and controls, consistent with our finding that NtsR1 and NtsR2 are expressed on distinct VTA cell populations (Fig. 18). No differences in NA expression of *D1* or *D2* were observed between groups (Fig. 25E). In sum, loss of VTA NtsR1 neurons results in structural and molecular adaptations in the mesolimbic DA system but does not abrogate the entirety of DA-mediated signaling that is required for survival.

4.4 Discussion

The lack of molecular markers to differentiate functionally heterogeneous and projection-specified VTA DA neurons has impeded understanding of how these neurons orchestrate behavior and body weight. A recent study reported that a subpopulation of VTA DA neurons

that project to the lateral septum can be identified by the transcription factor, *Neurod6*[32], however no additional markers of projection-specific VTA subsets have been identified. Here, we show that NtsR1 expression identifies a subset of VTA DA neurons that specifically projects to the ventral striatum and thus is the first molecular marker specific to mesolimbic DA neurons. Loss of this VTA NtsR1 population alters DA-mediated behaviors to protect against weight gain without compromising DA-mediated signaling necessary for survival[4]. Collectively, our data identify VTA NtsR1 neurons as important coordinators of energy intake and output behaviors that determine body weight.

Since Nts actions via NtsR1 and NtsR2 are implicated in control of distinct physiology, we hypothesized that these receptors might identify functionally distinct DA neurons. We therefore used Cre-mediated reporter mice to identify the cells expressing each receptor isoform, confirming that NtsR1 is almost exclusively expressed by DA neurons while NtsR2 is primarily expressed by non-DAergic neurons and astrocytes. Since NtsR1 is a G_q-coupled receptor[33], our findings indicate a mechanism for Nts to directly enhance the activation of NtsR1-expressing DA neurons, consistent with the established roles for NtsR1 in promoting DA release to the striatum and DA-mediated locomotor activity[34,35], anorexia[35], and reward[36,37]. Our data do not, however, rule out roles for the non-DAergic populations of NtsR2 cells to indirectly promote DA signaling. The TH-negative NtsR2 neurons found primarily within the ventromedial, GABA-rich portion of the VTA may disinhibit local DA neurons and promote their activation[37,38]. Nts may also act via the numerous NtsR2-expressing astrocytes within the VTA to indirectly modify DA-mediated behavior, consistent with reports of glia mediating NtsR2-dependent fear behavior[39]. Going forward, it will be important to define the neural circuits by which Nts engages these distinct NtsR1 and NtsR2-expressing cell types and their individual contributions to DA-mediated behaviors and energy balance.

In situ hybridization predicts a larger population of NtsR1-expressing neurons in the VTA than were identified using transgenic NtsR1^{Cre}-GFP mice (Allen Brain Atlas and Figure 1). This discrepancy may be due to low Cre expression common to transgenic lines, diminishing the intensity of Cre-mediated EGFP expression necessary to identify NtsR1 cells. Increasing the concentration of Cre-inducible transcripts can enhance recombination, and indeed, injecting *NtsR1^{Cre}* mice with AAV-GFP identifies additional NtsR1 neurons in the VTA. The AAV-identified VTA NtsR1-GFP neurons exclusively co-label with a subpopulation of TH cells, confirming that VTA NtsR1 neurons are a subset of all DA neurons (Fig. 26). In sum, these data suggest that the VTA NtsR1 neuronal population is larger than depicted in Fig. 18, but that subsequent AAV manipulations were able to transduce and modulate most VTA NtsR1 neurons (see further discussion in Chapter 6).

Although generalized depletion of VTA DA neurons does not alter locomotor activity or energy intake[40-42], ablation of the specific subset of VTA NtsR1 neurons altered both behaviors and prevented age-associated and diet-induced weight gain. Since aging and obesogenic environment are the two most significant instigators of human weight gain, our findings suggest roles for VTA NtsR1 neurons in countering the development of obesity. Intriguingly, the pronounced physical activity and energy expenditure of *NtsR1^{DTA}* mice appears to be the primary source of protection from weight gain, and is consistent with the established role for mesolimbic DA in regulating locomotor behavior. Although *NtsR1^{DTA}* mice eat slightly more than controls, their energy intake is only sufficient to compensate for elevated levels of activity and energy expenditure, and did not promote weight gain. Thus, mesolimbic VTA NtsR1 neurons are not essential drivers of feeding *per se*, but they coordinate energy intake required to support physical activity.

While open-circuit indirect calorimetry systems are widely accepted methods to analyze VO_2 and energy expenditure[53], methodological limitations should be taken into account when interpreting the data. For example, the metabolic chambers record food intake by detecting slight differences in weight of the food hopper as food is removed by the mouse during feeding. However, this does not ensure that all food removed from the hopper was consumed. Further, the analysis does not take into account the possibility of differences in digestion efficiency between *NtsR1*^{DTA} and control mice as not all ingested food may have been absorbed for energy. Differences in digestion could be addressed by assessing caloric content of fecal matter, which was not performed in this study. These two caveats together pose the possibility that food intake could be slightly overestimated. Another limitation is that O_2 and CO_2 concentrations were collected once for 3 minutes every 27 minutes over the duration each 4 day experiment. Thus, we can only assume that metabolism during the 3 minute collection period represents the entire 27 minute interval, which could overestimate or underestimate energy expenditure. To examine this, we plotted energy intake vs. expenditure for both groups at 4 and 16 weeks post-ablation (Fig. 20S, T). As long as no weight gain or loss occur, the slope of the distributions between intake and expenditure should be near 1.0. We found however, that the slopes were much less than 1.0 but similar between groups. A slope <1.0 suggests that the food intake exceeds energy expenditure, yet neither group gained weight during the study periods (data not shown), suggesting that either 1) food intake is overestimated due to lack of complete consumption or differences in digestion or 2) energy expenditure is underestimated, perhaps due to the assumption that energy expenditure during the 3 minute recording interval is representative of a larger time period, or a combination of both. However, the fact that the slopes did not vary significantly between ablated and control mice suggests that any error in estimating caloric intake or expenditure applies equally to both groups. Further, we used weight-adjusted ANCOVA analysis to detect significant increases in VO_2, VCO_2, and energy expenditure with VTA NtsR1 ablation (Fig. 20, 21), which eliminates the potential for erroneous

differences that can occur when metabolic parameters are simply divided by body weight[53]. Thus, although the absolute values for caloric intake and expenditure may vary by system, we firmly believe the differences detected between groups are valid.

Given that VTA NtsR1 neurons project to the NA, where DA release regulates the motivation for palatable foods[2,3], we were surprised that *NtsR1^{DTA}* mice did not differ from controls in their willingness to work for sucrose rewards. Furthermore, *NtsR1^{DTA}* mice demonstrated intact liking and wanting of palatable foods. These findings suggest that VTA NtsR1 neurons are not required for DA-mediated ingestive behavior, and that other non-NtsR1 expressing circuits exert this control. It may be argued, however, that *NtsR1^{DTA}* mice with low body weight and adiposity should display increased motivation for sucrose[28], and thus have a deficit in coordinating intake behavior to meet energy demands. This is consistent with our findings that *NtsR1^{DTA}* mice also lack the ability to adapt motivated intake in the face of satiety cues such as sucrose pre-feeding and leptin treatment[30]. Taken together, these data suggest that VTA NtsR1 neurons are not required for the execution of motivated food intake, but rather serve to tune it in response to peripheral energy needs.

VTA NtsR1 neurons must receive information about peripheral energy status to coordinate metabolic state and DA-mediated behavior. The adipocyte-derived hormone leptin modulates energy balance in part via the actions of Nts and NtsR1[25,43], and we demonstrate an obligate role of VTA NtsR1 neurons in this process. It is unlikely that VTA NtsR1 neurons are direct targets of leptin because VTA NtsR1- and leptin receptor (LepRb) expressing populations have distinct anatomical distributions and projection sites (Fig. 19 and [44]), suggesting that they comprise distinct neuronal populations. Leptin does, however activate LepRb neurons of the lateral hypothalamic area (LHA) that project to and release Nts into the VTA, promoting NtsR1-

dependent release of DA in the NA[25,45]. LHA LepRb neurons may thus be the neural hubs linking leptin action with Nts and DA signaling.

We used NtsR1 as a marker to identify VTA NtsR1 neurons, but it remains to be determined which signals from these neurons are critical for regulation of energy balance. VTA NtsR1 neurons may co-express both GABA and/or glutamate to modify activation of striatal targets. Nts action via NtsR1 expressed on VTA NtsR1 neurons may also contribute to DA-mediated behaviors. Indeed, the phenotype of *NtsR1*^{DTA} mice resembles whole-body NtsR1 knockout mice with hyperactivity, increased sucrose preference, and lack of adaptive response to leptin[25]. Together, these models argue that chronic disruption of Nts signaling via VTA NtsR1 neurons biases homeostasis toward energy expenditure. Perhaps the most important signal from VTA NtsR1 neurons may be DA itself. Although *NtsR1*^{DTA} mice exhibit substantial loss of VTA NtsR1 neurons and diminished DAergic projections to the NA, their intact AMPH-induced cFos confirms that at least some DA neurons remain and are functional. If anything, chronic loss of VTA NtsR1 neurons may lead to enhanced DA signaling. Indeed, mice lacking VTA NtsR1 neurons display increased general ambulatory behavior, but decreased AMPH-induced locomotor activity accompanied by stereotypy, a behavioral response typically associated with high-dose AMPH and ceiling-levels of DA efflux[46]. We thus speculate that loss of VTA NtsR1 neurons leads to increased striatal DA signaling, and AMPH further increases DA to levels that promote stereotypy instead of augmenting ambulatory activity. Numerous mechanisms could enhance DA action, such as altered balance of D1/D2 protein expression or impaired DAT kinetics, and will be important to define in the future.

Our data indicate that VTA NtsR1 neurons play an important role in preventing diet-induced obesity, hence VTA NtsR1 neurons may represent a novel cellular target for preventing and treating human obesity. Ablation of neurons is not a feasible therapeutic strategy, thus it

will be imperative to identify pharmacological means to selectively modulate VTA NtsR1 neurons to promote behaviors that prevent weight gain. Previous work demonstrates that pharmacologic treatment of Nts or Nts agonists in the VTA suppresses operant-reinforced feeding[47] and enhances locomotor activity in an NtsR1-dependent manner[19,34]. Conversely, other studies showed suppression of locomotor activity when Nts was applied directly to the NA[14], underscoring the need for circuit-specific interventions. Although developmental loss of NtsR1 in NtsR1 knockout mice promotes palatable food intake and weight gain[25], our current data show that selective ablation VTA NtsR1 neurons in adult mice protects them from obesity, suggesting that pharmacological modulation of Nts signaling may be useful in adults with established VTA NtsR1 DA circuits. Going forward, it will be important to define how Nts and other signals engage VTA NtsR1 neurons to identify strategies that prevent obesity

4.5 Methods

Reagents: Recombinant leptin was purchased from the National Hormone and Peptide Program (Torrance, CA). The dopamine receptor-1 antagonist, SCH233990, was purchased from Sigma. Amphetamine hydrochloride was from Cayman Pharmaceuticals.

Mice: Transgenic *NtsR1*^{Cre} mice were purchased from the Mutant Mouse Regional Resource Center at UC Davis (B6.FVB(Cg)-Tg(NtsR1-cre)GN220Gsat/Mmucd, Stcok number 030648-UCD). *NtsR2*^{Cre} mice were generated via homologous recombination (knock-in) methods described previously[48]. To visualize NtsR1 and NtsR2-expressing neurons, *NtsR1*^{Cre} and *NtsR2*^{Cre} mice were bred to a Cre-inducible Rosa$^{eGFP\text{-}L10a}$ reporter line, generating mice that express GFP selectively in NtsR1 and NtsR2 neurons (*NtsR1*^{Cre}*;GFP* and *NtsR2*^{Cre}*;GFP*). Mice were bred and housed in a 12h light/12h dark cycle and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Generation of NtsR2^{Cre} Knock-In Mice: An *NtsR2^{cre}* targeting vector was generated by inserting an IRES-Cre sequence between the stop codon and the polyadenylation site of the sequence encoding the 3' end of the mouse *NtsR2* gene, with an *frt*-flanked Neo cassette inserted upstream of the IRES-Cre. The linearized targeting vector was electroporated into mouse R1 embryonic stem (ES) cells (129sv background) and cells were selected with G418. DNA from ES cell clones was analyzed via qPCR for loss of homozygosity using Taqman primer and probes for the genomic *NtsR2* insertion site (Forward: ACCCATCAGATAAGCCATGC, Reverse: GTGGGAAGTTGAGGGCAG, Probe: GTCTAAGCGGACCTACTGACCCA). *NGF* was used as a copy number control[49]. Putative positive ES clones were expanded, confirmed for homologous recombination by Southern blot and injected into mouse C57BL/6 blastocysts to generate chimeras. Chimeric males were mated with C57BL/6 females (Jackson Laboratory), and germline transmission was determined initially via progeny coat color, then confirmed via conventional PCR for IRES-Cre.

Genotyping: *NtsR1^{Cre}* and *NtsR2^{Cre}* mice were bred to C57BL/6J mice to maintain the lines, and progeny were genotyped via standard PCR (*NtsR1^{Cre}*: F: GACGGCACGCCCCCCTTA, R: CGGCAAACGGACAGAAGCATT, *NtsR2^{Cre}*: CCGTGTCTTCCTTCAGA, R: CTACACCTTGGTTGCACAGG).

Surgery: Mice received a pre-surgical injection of carprofen (5mg/kg *s.c.*) and were anesthetized with 3-4% isoflurane/O_2 in an induction chamber before being placed in a stereotaxic frame (Kopf). Under 1-2% isoflurane, access holes were drilled in the skull allowing a guide cannula with stylet (PlasticsOne) to be lowered into the brain target area. After 7 min to allow for AAV absorption, the injector and cannula were removed from the skull and the incision was closed using Vet Bond. To generate *NtsR1^{GFP}* and *NtsR1^{DTA}* study mice, 8-10 wk old *NtsR1^{Cre};GFP* males received bilateral injections of either 150 nL of Cre-inducible AAV-GFP

(rAAV2/hSvn-DIO-eGFP, University of North Carolina Vector Core) or AAV-DTA (lox-mCherry-loxDTA-WPRE-AAV, serotype 10), which expresses the cytotoxic Subunit A of Diptheria Toxin in the presence of Cre, into the VTA (A/P: -3.2, M/L: +/-0.48, D/V: -4.65). *NtsR1*^{GFP} and *NtsR1*^{DTA} mice were monitored weekly for body weight after surgery, but did not undergo analysis until 4 wks post-surgery to allow DTA-mediated cell death to occur. We generated and studied *NtsR1*^{GFP} = 7, *NtsR1*^{DTA} =15 and *NtsR1*^{GFP} *HF*=10, *NtsR1*^{DTA} *HF*=13. Mice were only included in the final study data if injections were localized to, and contained within the VTA which was determined by specific lack of GFP expression in the VTA. After post-hoc examination, 2/15 *NtsR1*^{DTA} and 3/13 *NtsR1*$^{DTA\text{-}HF}$ mice were excluded due to unilateral targeting and/or spread of the injection site to the substantia nigra (SN), leaving *NtsR1*^{GFP} = 7 and *NtsR1*^{DTA} =13; *NtsR1*^{GFP} *HF*=10 and *NtsR1*^{DTA} *HF*=10.

To visualize NtsR2 neurons, adult male and female *NtsR2*^{Cre}*;GFP* were bilaterally injected with 1uL FlpO adenovirus (Vector Biolabs) into the lateral ventricles in accordance with the atlas of Paxinos and Franklin[50]: A/P: -0.22, M/L: +/- 1.0, D/V: -2.0. Mice were perfused 10 days after surgery to permit sufficient time for FlpO-mediated excision of the *frt*-flanked Neo cassette and GFP expression. For tracing studies, *NtsR1*^{Cre} mice were injected unilaterally in the VTA with 75-100 nL of an anterograde cre-inducible adenovirus expressing a synaptophysin-mCherry fusion protein, Ad-syn-mCherry[25], (Martin Myers, University of Michigan) using the same coordinates described above. Mice recovered for 7-10 days after surgery to allow for synaptophysin-mCherry expression at pre-synaptic terminals.

Metabolic Profiling: At 4, 8 and 16 wk post-surgery the mice were analyzed for body composition using and NMR-based instrument (Minispec mq7.5, Bruker Optics). At 4 and 16 wk post-surgery, mice were placed in TSE cages for metabolic phenotyping (PhenoMaster, TSE Systems). After 24 hours of acclimation, mice were continuously monitored for food and water

intake, locomotor activity, and energy expenditure over 4 days. Ambient temperature was maintained at 20-23˚C and the airflow rate through the chambers was adjusted to maintain an oxygen differential around 0.3% at resting conditions. The O_2 and CO_2 sensors were calibrated to gas mixtures with verified gas concentrations. All air entering the system was filtered and dried to avoid variation in gas partial pressure due to moisture. O_2 and CO_2 concentrations and gas flow rates were measured over a 3 minute period from each cage individually, every 27 minutes for the duration of the run. The system consisted of 8 air-tight cages containing singly-housed study animals and an empty cage from which gas concentrations were used as a reference to calculate oxygen consumption (VO_2), carbon dioxide production (VCO_2), energy expenditure, and respiratory exchange ratio (RER) as described below.

Metabolic Calculations:

$\mathbf{VO_2} = (Flow_{in} * [O_2]_{in}) - (Flow_{out} * [O_2]_{out})$

$\mathbf{VCO_2} = (Flow_{in} * [CO_2]_{in}) - (Flow_{out} * [CO_2]_{out})$

In these equations, $[O_2]_{out}$, $[CO_2]_{out}$ and $Flow_{out}$ are measured from air exiting the experimental cage, while $[O_2]_{in}$ and $[CO_2]_{in}$ are measured from the reference cage. Note that although gas concentrations from both reference and experimental cages are measured after they exit the cage, no gas exchange has occurred in the reference cage concentrations can be used for the "in" terms for all cages. The only term not measured directly is $Flow_{in}$, which can be solved for using the Haldane correction, based on the principle that the nitrogen concentration if air going into the cage should equal air exiting the cage as nitrogen is physiologically inert. Taking this into account:

Haldane Correction: $Flow_{in} * (1-([O_2]_{in} + [CO_2]_{in})) = Flow_{out} * (1-([O_2]_{out} + [CO_2]_{out}))$

Thus, $Flow_{in}$ can be solved for and used to calculate VO_2 and VCO_2.

RER= VCO_2/VO_2 →indicates whether glucose or fatty acids are preferentially used as energy substrates. Fatty acid oxidation yields a ratio of 0.7 while glycolysis is 1.0.

Energy Expenditure was calculated using the Weir equation:

Metabolic rate (kcal/hr) = (3.815+1.232*RER) * VO_2

All parameters were calculated by the TSE system software.

Sucrose Preference: *NtsR1^{DTA}* and *NtsR1^{GFP}* mice were given two identical 50 mL sipper bottles in their home cages for 4 consecutive days. On days 1-2, both bottles were filled with water to acclimate the animals to the test environment. On days 3-4, one bottle was switched to 0.5% sucrose while the other contained water. The bottle positions were rotated daily to avoid position bias, and the volume consumed from each bottle was measured at the same time each day. To assess leptin-induced changes in sucrose preference, animals were re-tested one month later for 6 consecutive days. On days 1-2, animals received once daily sham *i.p.* injections while both bottles contained water. On day 3, one bottle was switched to 0.5% sucrose. Mice were treated with PBS on days 3-4 and leptin (5mg/kg *ip*) on days 5-6. All injections were administered at the onset of the dark cycle when most food and water intake naturally occurs. Data are reported as sucrose preference, which is the percentage of sucrose consumed out of total liquid consumed.

Operant Testing: Standard Testing: *NtsR1^{DTA}* and *NtsR1^{GFP}* were trained to nose-poke for unflavored 20 mg sucrose pellets (TestDiet 1811555) using operant-responding chambers (Med Associates). As previously described[30], mice were food restricted to 90% of their body weight and trained on a fixed ratio-1 (FR1) schedule until they achieved 75% response accuracy

with ≥20 rewards earned on 3 consecutive days. Training sessions were terminated after 1 hr or when the animal had earned 50 rewards. Mice achieving these criteria were then switched to *ad lib* food and trained on an FR5 schedule for 3 consecutive days. On test days, mice were subject to a progressive ratio (PR) schedule were PR=[$5e^{(R*0.2)}$]-5 with R=number food rewards earned+1[51]. Thus, the number of correct responses needed to earn a sucrose reward increases as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 *etc*. The PR breakpoint was recorded as the highest ratio completed for each 1 hr test session. Mice were tested until they achieved stable PR which was defined as <10% variation in rewards earned over 3 consecutive sessions. *Sucrose pre-feeding:* After achieving stable PR, mice were given *ad lib* access to 3g of sucrose pellets in their home cages overnight. The following morning, they were re-tested on the PR schedule. *Leptin Treatment:* Mice with stable PR responses were injected with PBS or leptin (5mg/kg, *ip*) on different days and tested one hour later.

Elevated Plus Maze: Mice were assessed for anxiety-like behavior using an elevated plus maze (EPM) as previously described[52]. Briefly, the EPM apparatus was custom-built based on plans from ANY-maze (www.anymaze.com, Stoelting Co.) and mice were given free access to the open and closed arms for 5 minutes. Their behavior was recorded using a digital CCD camera and the percentages of time spent in the open and closed arms were analyzed using Topscan automated video tracking software (Clever Sys).

Open Field Locomotor Activity: Open field locomotor activity was assessed using a digital CCD camera and video-tracking software (Clever Sys)[52]. Mice were tested on 3 separate days; each day, mice were placed in the boxes to acclimate, followed by an *i.p.* injection of PBS 30 min later. At 60 min, mice received either a second injection of PBS, or SCH23390 (0.1mg/kg) or amphetamine (4mg/kg) and recorded for another 60 minutes.

Immunohistochemistry and Immunofluorescence: Mice were treated with a lethal dose of *ip* pentobarbital followed by transcardial perfusion with 10% neutral-buffered formalin (Fisher). Brains were removed, post-fixed in 10% formalin overnight at 4˚C, dehydrated with 30% sucrose in PBS for 2-3 days, and sectioned into 30 µm slices using a sliding microtome (Leica). Brain sections were then analyzed by immunofluorescence or immunohistochemistry as previously described[25,48]. For characterization of NtsR1 and NtsR2 expression, sections from *NtsR1^{Cre};GFP* and *NtsR2^{Cre};GFP* mice were stained with chicken anti-GFP (1:2000, Abcam), mouse anti-TH (1:1000, Millipore), or rabbit anti-S100 (1:1000, Abcam), followed by incubation with species-specific secondary antibodies conjugated to AlexaFluor 488 or 568 fluorophores (Life Technologies or Jackson ImmunoResearch). For ablation studies, *NtsR1^{DTA}* and *NtsR1^{GFP}* mice were treated with PBS or amphetamine (4mg/kg) 90 minutes prior to perfusion, and brain sections were stained for cFos (1:500, goat, Santa Cruz) with secondary detection via DAB (Life Technologies), followed by immunofluorescent detection of GFP, TH, or DAT (1:1000, Millipore) as described above. Brains were analyzed using an Olympus BX53 fluorescence microscope outfitted with transmitted light to analyze DAB-labeled tissue, as well as FITC and Texas Red filters. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe). For quantification and colocalization of NtsR1 and NtsR2 neurons with TH, five representative sections spanning the entire VTA were selected from each mouse, from which all GFP and/or TH positive neurons were counted.

Gene Expression: At 16 weeks post-surgery, *NtsR1$^{DTA\text{-}Uni}$* (n=17) and *NtsR1$^{GFP\text{-}Uni}$* (n=7) were deeply anesthetized with sodium pentobarbital and tissue from the injected and uninjected sides of the VTA and NA were separately microdissected. Tissue was immediately snap frozen on dry ice and stored at -80˚C for later processing. RNA was extracted using Trizol (Invitrogen) and 200 ng samples were converted to cDNA using the Superscript First Strand Synthesis

System for RT-PCR (Invitrogen). Sample cDNAs were analyzed in triplicate via quantitative RT-PCR for gene expression using TaqMan reagents and an ABI 7900 (Applied Biosystems) at the MSU Genomics Core. With *GAPDH* expression as an internal control, relative mRNA expression values were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to the uninjected side of each mouse. To verify targeting, *NtsR1*$^{DTA\text{-}Uni}$ mice were considered sufficiently ablated if the fold change in NtsR1 expression was less than 1 standard deviation below the mean NtsR1 fold change in AAV-GFP-injected mice. By this criterion, 7 of 15 *NtsR1*$^{DTA\text{-}Uni}$ mice were deemed insufficiently ablated and were excluded from the analysis.

Statistics: Student's t-tests and 2-way ANOVA were calculated using Prism 6 (GraphPad). For all metabolic data, analysis of covariance (ANCOVA) was computed in SPSS 22 (IBM). Body weight was analyzed as a covariate to correct for any inherent differences it may have on metabolism[53]. All data were tested for homogeneity of regression, independence of the covariate (body weight), and linearity of regression prior to running the ANCOVA. For all data, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

APPENDIX

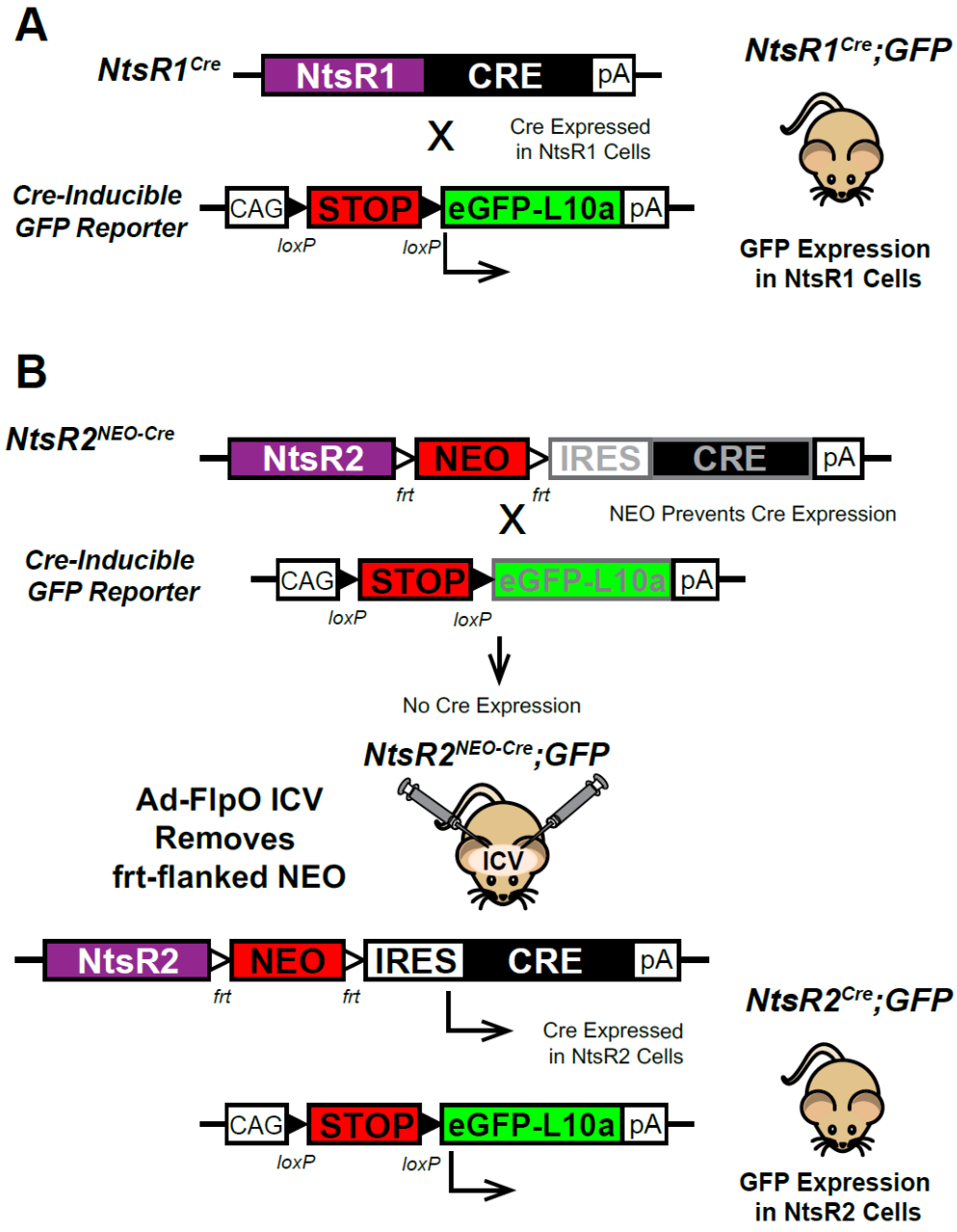

Figure 17. Generation of *NtsR1*$^{Cre;}$*GFP* and *NtsR2*^{Cre}*;GFP* reporter mice to visualize NtsR1 and NtsR2 neurons in the VTA. A) Commercially-available transgenic *NtsR1*^{Cre} mice were bred to a Cre-inducible GPP reporter line to generate mice expressing GFP selectively in NtsR1 neurons (*NtsR1*^{Cre}*;GFP* mice). **B)** *NtsR2*$^{NEO\text{-}Cre}$ mice were created by inserting an IRES-Cre sequence downstream of an *frt*-flanked NEO cassette directly into the *Ntsr2* locus using homologous recombination. *NtsR2*$^{NEO\text{-}Cre}$ mice were then crossed to a Cre-inducible GFP reporter line, however GFP is not expressed because the NEO cassette prevents Cre expression. Thus, adult *NtsR2*$^{NEO\text{-}Cre}$*;GFP* mice were injected with an adenovirus expressing FlpO recombinase ICV to remove the *frt*-flanked NEO cassette, resulting in Cre and therefore GFP expression in NtsR2-positive cells (*NtsR2*$^{NEO\text{-}Cre}$*;GFP* mice).

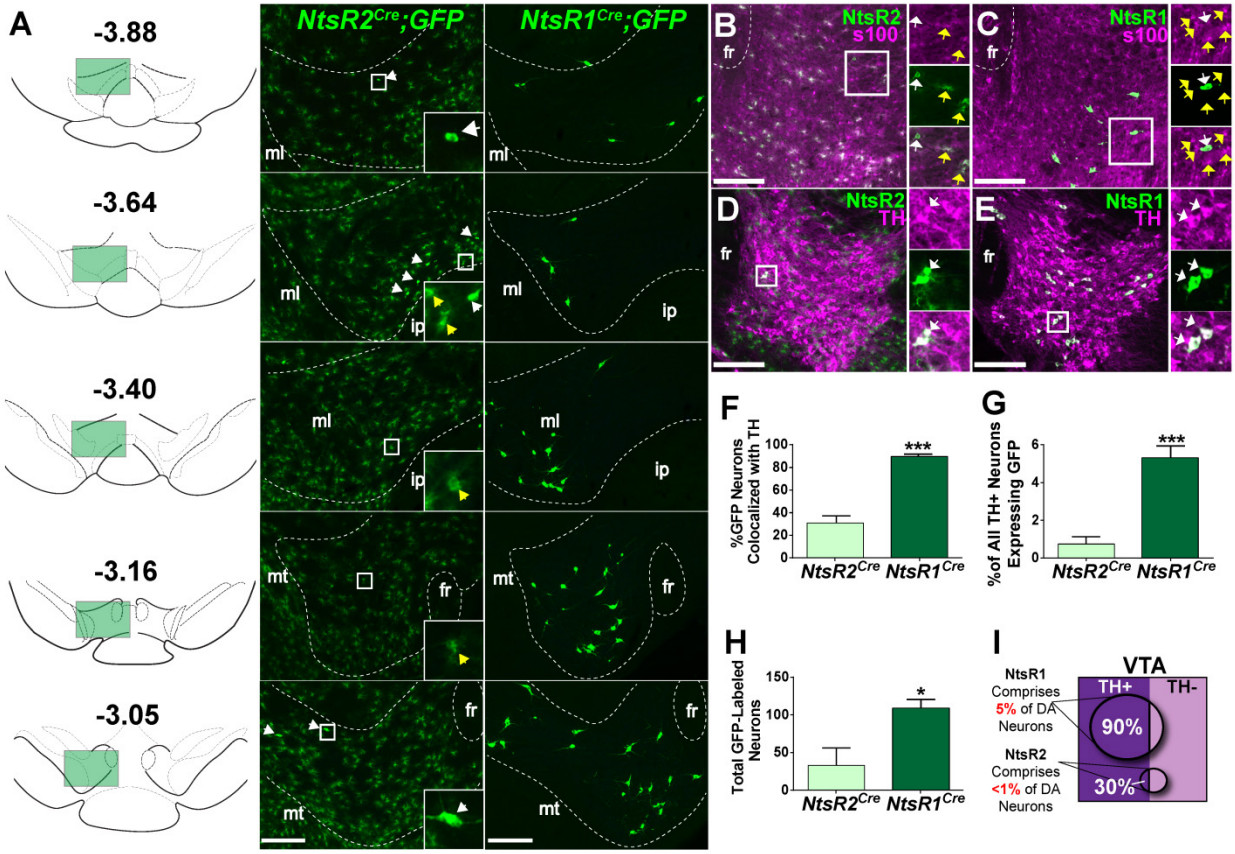

Figure 18. Distribution and neurochemical phenotype of VTA NtsR1 and NtsR2 neurons. VTA NtsR1and NtsR2 neurons were visualized by crossing *NtsR1^{Cre}* and *NtsR2^{Cre}* mice to a Cre-inducible GFP reporter line. *See Fig. 17 for more details*. **A)** Rostrocaudal distribution of VTA NtsR1 and NtsR2 neurons, with bregma positions from the mouse brain atlas of Paxinos and Franklin. White arrows denote neurons, yellow arrows denote glial cells. **B)** Colocalization of glial cell marker S100 (purple) in the VTA with NtsR2 cells (green) and **C)** NtsR1 cells (green). **D)** Colocalization of tyrosine hydroxylase (TH, purple) in the VTA with NtsR2 cells (green) and **E)** NtsR1 cells (green). **F)** Percentage of NtsR1 and NtsR2 neurons that colocalize with TH. **G)** Percentage of TH positive neurons that colocalize with NtsR1 or NtsR2. **H)** Total number of GFP-identified NtsR1 and NtsR2 neurons in the VTA. **I)** Schematic depicting the distribution of VTA DA and non-DA neurons that co-express NtsR1 and NtsR2. *NtsR1^{Cre};GFP* n=4, *NtsR2^{Cre};GFP* n=6. Data represent mean ± SEM, *p < 0.05, ***p < 0.001 determined by unpaired t-test. Scale bars = 100 µm. *ml = medial lemniscus; ip = interpeduncular nucleus; fr = fasciculus retroflexus.*

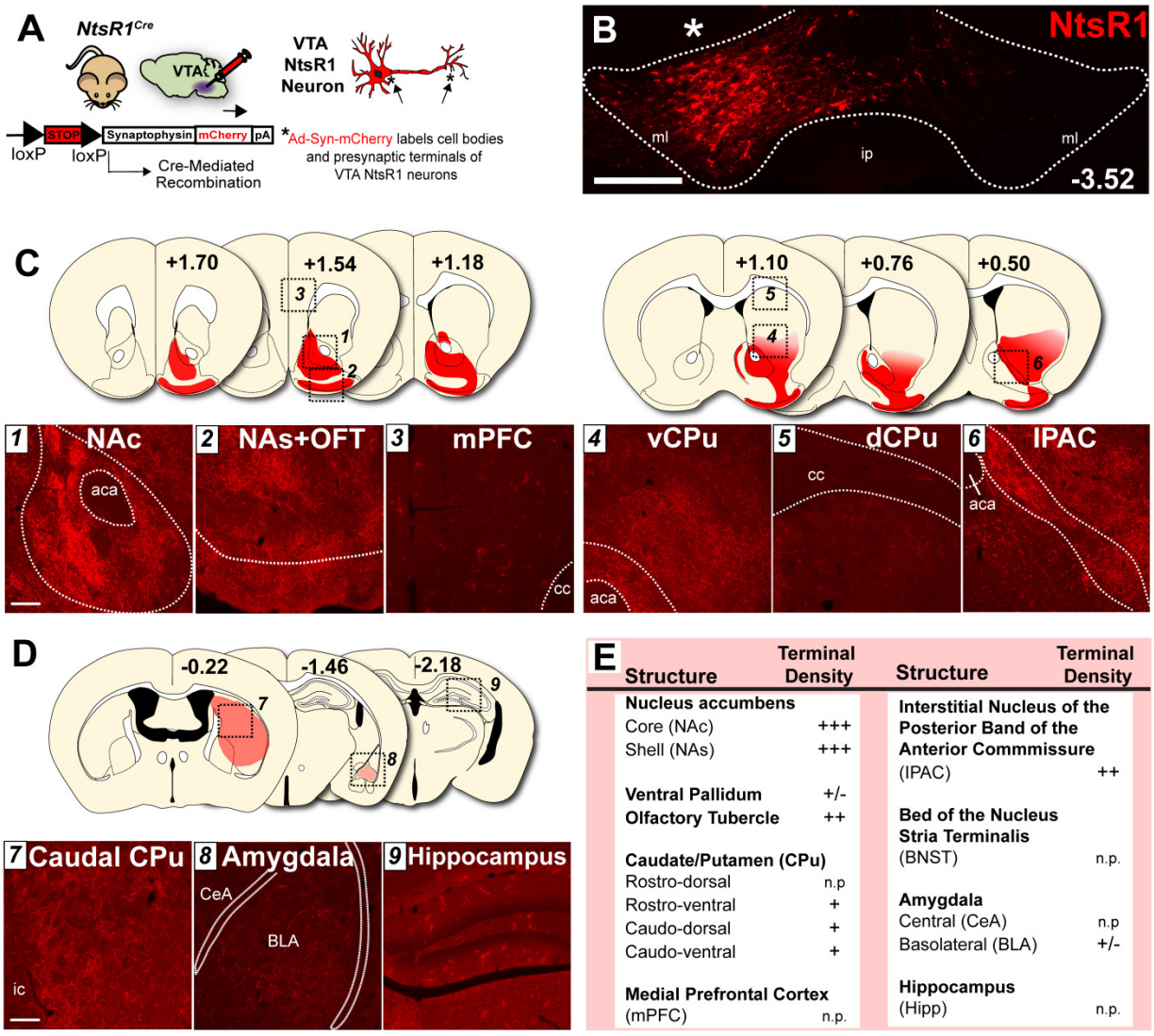

Figure 19. VTA NtsR1 neurons project to the ventral striatum. A) *NtsR1^{Cre}* mice were unilaterally injected in the VTA with Ad-Syn-mCherry, resulting in expression of a synaptophysin-mCherry fusion protein in the cell bodies and presynaptic terminals of VTA NtsR1 neurons (n=5). **B)** VTA injection site (left, asterisk) with mCherry-labeled NtsR1 cell bodies. Scale bar = 200 µm. **C)** Major efferent targets of VTA NtsR1 neurons. **D)** Minor efferent targets of VTA NtsR1 neurons. Numbered dashed-boxes in coronal schematic images correspond to microscopy images below. Bregma positions are according to the mouse brain atlas of Paxinos and Franklin. Scale bars in c, d = 100 µm. **E)** Table summarizing relative NtsR1 terminal density across multiple brain areas with emphasis on previously established VTA efferents. Note: n.p.= not present. *aca=anterior commissure, cc=corpus callosum, ic=internal capsule, ml=medial lemniscus, ip=interpeduncular nucleus, CeA= central amygdala, BLA= basolateral amygdala.*

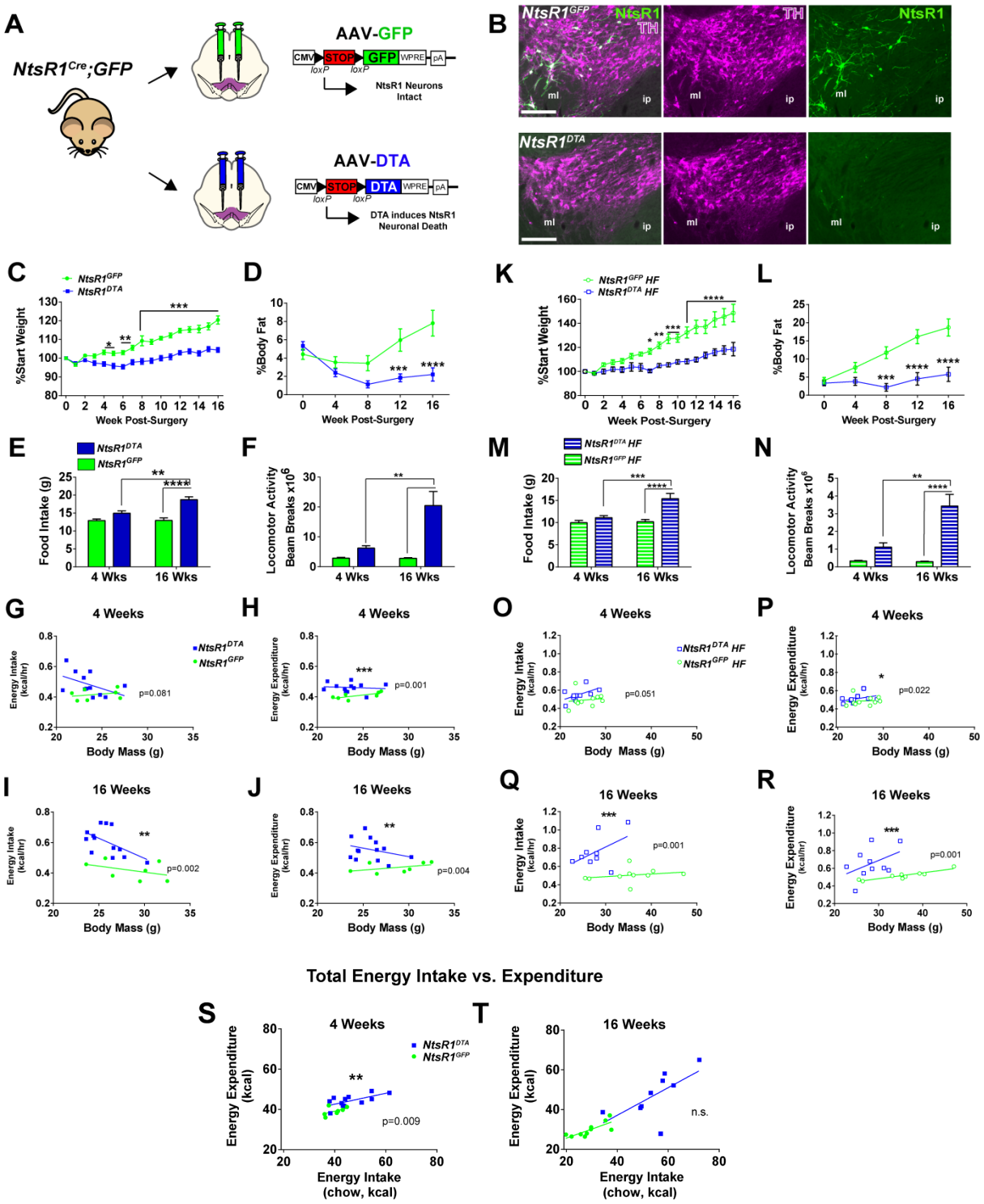

Figure 20. Loss of VTA NtsR1 neurons disrupts energy balance. A) 8 wk old male *NtsR1^{Cre};GFP* mice received bilateral injections of AAV-DTA or AAV-GFP in the VTA. **B)** Images show representative NtsR1-GFP (green) and TH (purple) expression from *NtsR1^{GFP}* and *NtsR1^{DTA}* mice two weeks after surgery. Scale bars =200µm. *ml=medial lemniscus, ip=interpeduncular nucleus.*

Figure 20 (*cont'd*). C-J) Metabolic assessment of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice on chow diet (*NtsR1*^{GFP} n=7, and *NtsR1*^{DTA} n=13). **C)** Percentage change in body weight from starting weight and **D)** percentage of body fat determined via NMR-based instrument. **E)** Chow intake and **F)** total locomotor activity as measured in TSE metabolic cages. **G)** Weight- adjusted energy intake and **H)** energy expenditure at 4 wk post-surgery and **I)** weight-adjusted energy intake and **J)** energy expenditure measured at 16 wk post-surgery. **K-R)** Metabolic assessment of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice on HF diet (*NtsR1*^{GFP}*HF* n=10 and *NtsR1*^{DT} *HF* n=10). **K)** Percentage change in body weight from starting weight, **L)** percentage of body fat, **M)** HF diet intake and **N)** total locomotor activity. **O)** Energy intake and **P)** energy expenditure at 4 wk post-surgery, and **Q)** energy intake and **R)** energy expenditure measured at 16 wk post-surgery, normalized to body mass. **S)** Total energy intake vs. estimated expenditure over 48 hours at 4 weeks (*NtsR1*^{GFP}: y=0.317x+26.43, *NtsR1*^{DTA}: y= 0.271x+31.74) and **T)** 16 weeks post-ablation (*NtsR1*^{GFP}: y=0.456x+16.48, *NtsR1*^{DTA}: y= 0.695x+9.298) . For **B-E** and **J-M**, graphed data represent mean ± SEM, *p < 0.05, **p < 0.01, ****p < 0.0001 analyzed by two-way ANOVA. For scatterplots in **G-J and O-R** data were analyzed using ANCOVA to account for body weight as a covariate. **S-T** were analyzed by linear regression. *See Fig. 21 for water intake and additional metabolic parameters.*

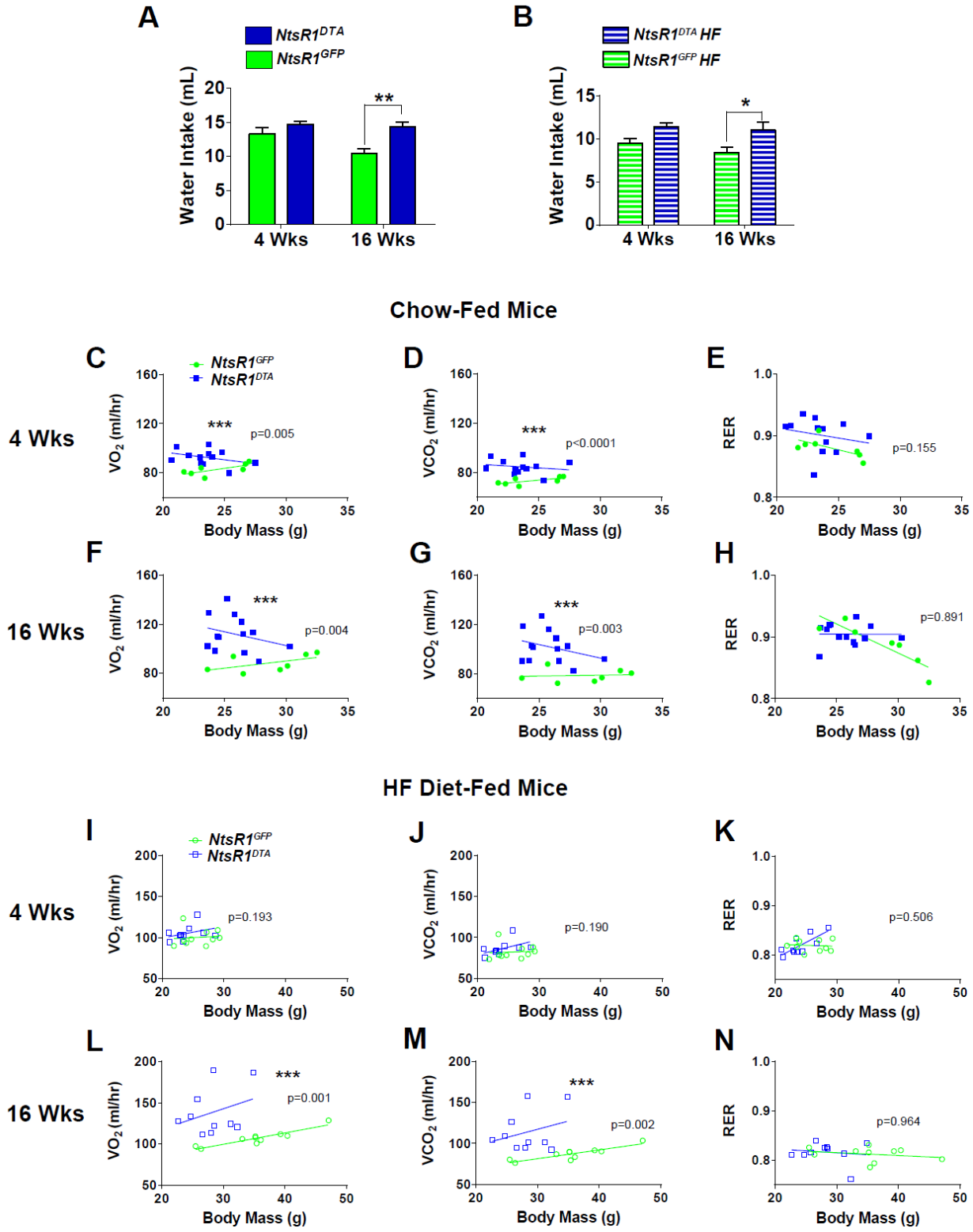

Figure 21. Water intake and additional metabolic parameters in mice lacking VTA NtsR1 neurons. Fluid intake and metabolic parameters were assessed over 4 days in TSE metabolic cages in *NtsR1*^{GFP} and *NtsR1*^{DTA} mice at 4 and 16 wk post-surgery.

Figure 21 (cont'd). Water consumption in **A)** chow-fed and **B)** HF diet-fed mice. Data represent mean ± SEM, analyzed by two-way ANOVA. **C)** oxygen consumption (VO_2), **D)** carbon dioxide production (VCO_2) and **E)** respiratory exchange ratio (RER) at 4 weeks-post ablation in chow-fed animals. **F)** VO_2, **G)** VCO_2, and **H)** RER in chow-fed mice 16 weeks post-ablation. **I)** VO_2, **J)** VCO_2, and **K)** RER in HF diet-fed mice at 4 weeks. **L)** VO_2, **M)** VCO_2 and **N)** RER in HF-diet fed mice 16 weeks post-ablation. All metabolic data were analyzed via ANCOVA to adjust for body weight as a covariate. (*NtsR1*^{GFP} n=7, *NtsR1*^{DTA} n=13, *NtsR1*^{GFP}*HF* n=10 and *NtsR1*^{DTA} *HF* n=10).

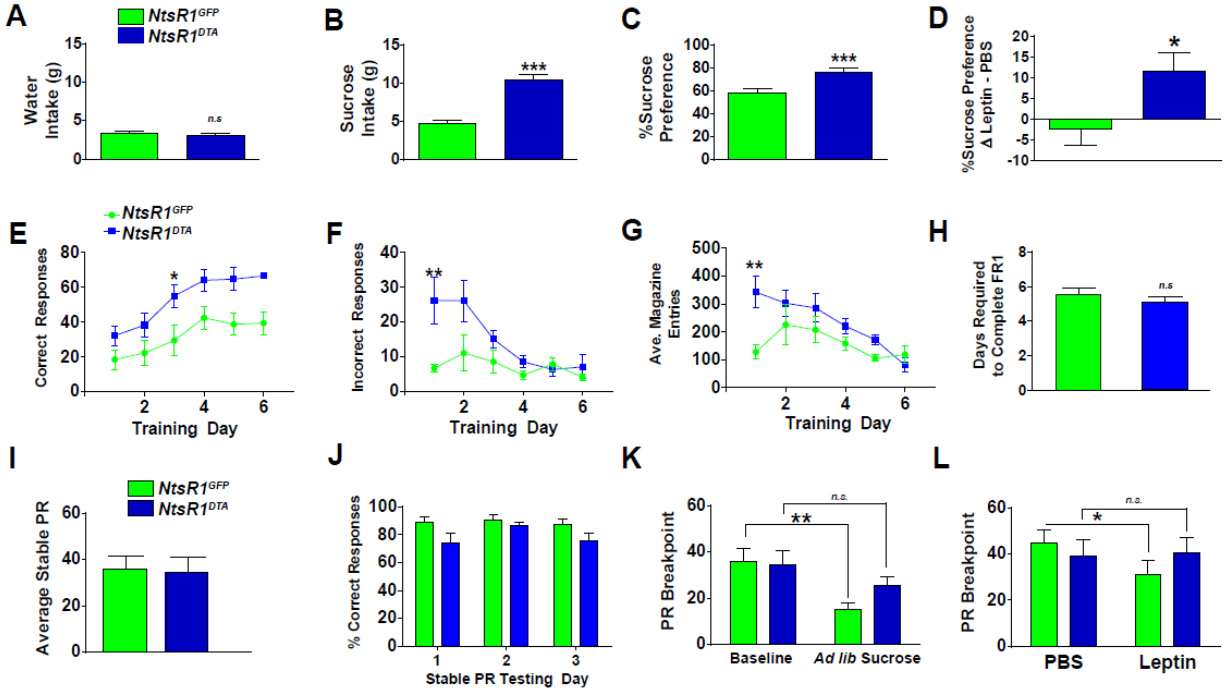

Figure 22. VTA NtsR1 neurons modulate hedonic and motivated ingestive behavior. **A)** Water intake, **B)** 0.5% sucrose intake and **C)** sucrose preference in untreated *NtsR1*^{GFP} and *NtsR1*^{DTA} mice over 4 days. **D)** Sucrose preference while mice received PBS or leptin (5mg/kg, *i.p.*) at the onset of the dark cycle. Graphed data represent the difference in average sucrose preference during PBS and leptin treatments. *NtsR1*^{GFP} n=7, and *NtsR1*^{DTA} n=13, n.s.=not significant, *p < 0.05, ***p < 0.001 by unpaired t-test. **E-H)** *NtsR1*^{GFP} and *NtsR1*^{DTA} mice were trained to nose-poke for sucrose pellets and assessed for response accuracy and magazine entries during FR1 training. **E)** Number of correct responses, **F)** number of incorrect responses **G)** number of magazine entries and **H)** days required to complete FR1 training. **I-L)** *NtsR1*^{GFP} and *NtsR1*^{DTA} mice were tested on a PR schedule until the number of rewards earned varied <10% over 3 consecutive days. **I)** Average stable PR breakpoint and **J)** percentage of correct responses over the three stable PR days. **K)** PR breakpoint was assessed after animals received *ad lib* access to sucrose rewards the night before testing. **L)** *NtsR1*^{GFP} and *NtsR1*^{DTA} mice were tested one hour after treatment with either PBS or leptin (5mg/kg, *i.p.*). *NtsR1*^{GFP} n=6, and *NtsR1*^{DTA} =10, n.s.=not significant, *p < 0.05, **p < 0.01, analyzed via repeated measures two-way ANOVA with Bonferroni post-tests. All graphed data represent mean ± SEM.

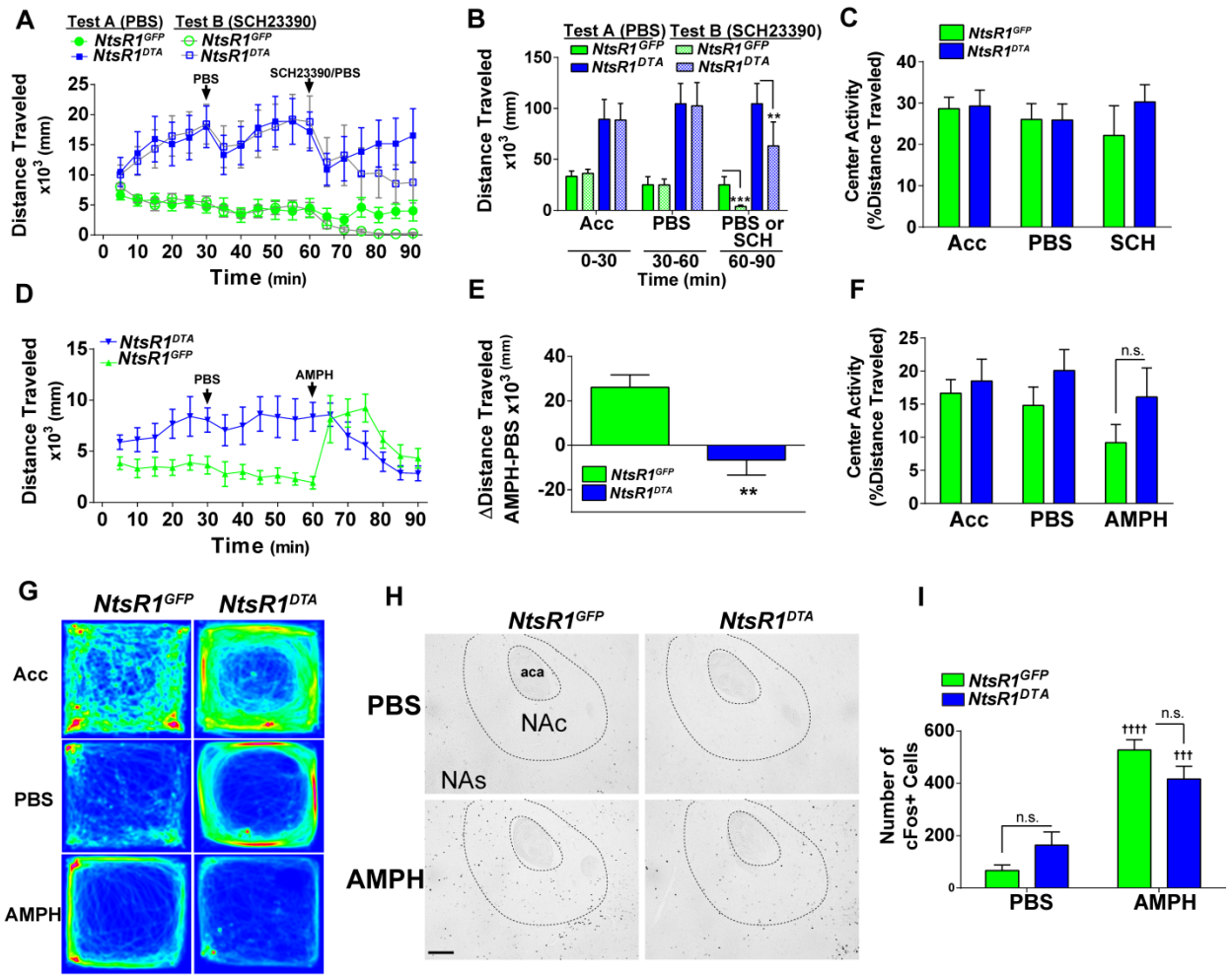

Figure 23. Loss of VTA NtsR1 neurons alters response to amphetamine. A) Locomotor activity of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice in open field boxes during acclimation (Acc), treatment with PBS, and either PBS (Test A) or D1R antagonist SCH23390 (0.1mg/kg, *i.p.*) (Test B). Data represent average distance traveled per 5 min interval ± SEM (*NtsR1*^{GFP} n=6, and *NtsR1*^{DTA} n=10). **B)** Mean distance traveled during treatment period ± SEM, analyzed by two-way ANOVA. **C)** Percentage of distance traveled in the center zone of boxes over each testing period. **D)** Open field locomotor activity of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice after treatment with PBS and AMPH (4mg/kg, *i.p.*). Data represent average distance traveled per 5 min interval ± SEM (*NtsR1*^{GFP} n=6, and *NtsR1*^{DTA} n=10). **E)** Mean difference between locomotor activity after AMPH treatment relative to PBS, **p=0.003. **F)** Percentage of distance traveled in the center zone over each period. *See Fig. 24 for additional anxiety data.* **G)** Representative heat maps demonstrating mouse location during acclimation, PBS, and AMPH treatment. **H)** cFos expression in the NA (-1.34 bregma) of *NtsR1*^{GFP} and *NtsR1*^{DTA} treated with PBS or AMPH 90 minutes prior to perfusion*). aca = anterior commissure; NAc = nucleus accumbens core; NAs = nucleus accumbens shell.* **I)** Number of cFos positive (cFos+) cells in NA. Graphed data represent mean ± SEM, analyzed by two-way ANOVA. ††† p<0.001, †† p<0.001, indicates significant difference between PBS and amphetamine treatment within each group, (*NtsR1*^{GFP} PBS n=3, AMPH=3; *NtsR1*^{DTA} PBS n=3, AMPH n=5).

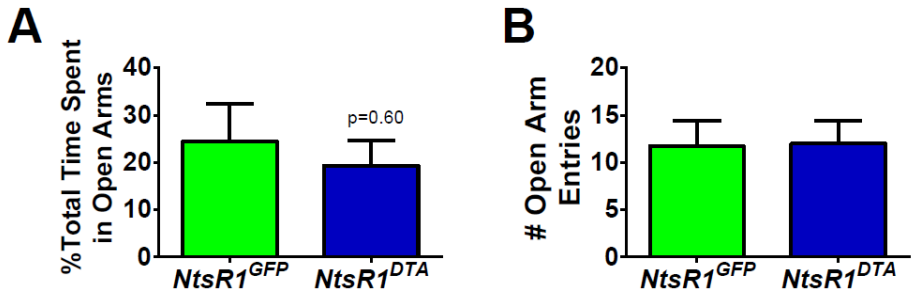

Figure 24. Anxiety-like behavior assessed via elevated-plus maze. No significant differences were found between groups in **A**) percentage of time spent on open arms or **B)** number of open arm entries (*NtsR1*^{GFP} n=4, *NtsR1*^{DTA} n=7).

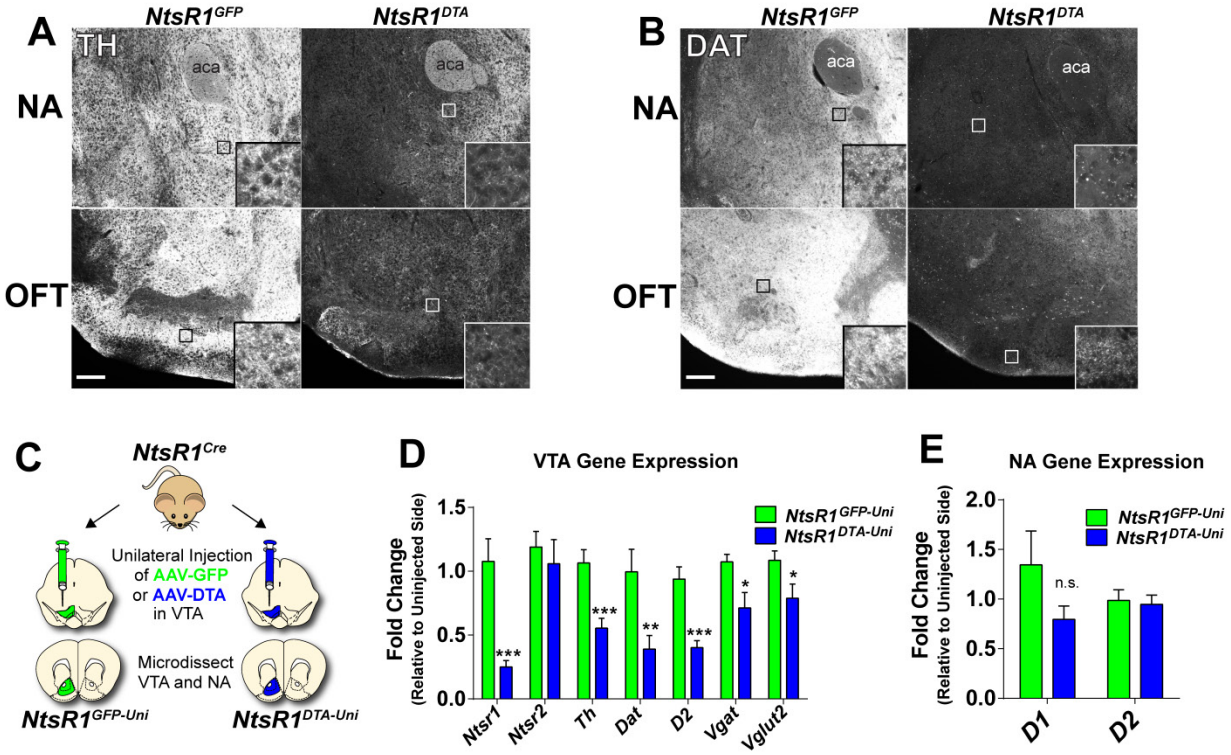

Figure 25. Ablation of VTA NtsR1 neurons decreases markers of DA signaling. Post-hoc analysis of **A)** tyrosine hydroxylase (TH) and **B)** dopamine transporter (DAT) immunoreactivity in the NA and OFT (-1.50 bregma) of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice perfused 7-11 months after surgery. Scale bars = 100 µm. White boxes identify digitally enlarged areas from each image. **C)** *NtsR1*^{Cre} mice were injected unilaterally with AAV-DTA to ablate VTA NtsR1 neurons or AAV-GFP as a control. The VTA and NA were microdissected separately from each side of the brain and expression of DA-related genes was assessed at 16 wk post-surgery in the **D)** VTA and **E)** NA. Fold change was determined for each animal relative to the un-injected side. Data are expressed as mean ± SEM (*NtsR1*^{GFP} n=6-7, *NtsR1*^{DTA} n=8-9). Significant differences were determined by unpaired t-test *p < 0.5, **p < 0.1, ***p < 0.001. *aca* = *anterior commissure*.

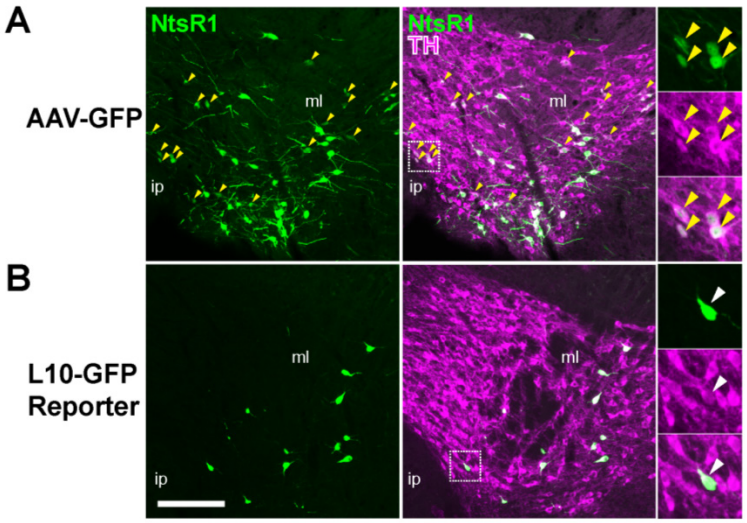

Figure 26. Virally-induced vs. endogenous GFP reporter expression for identification of VTA NtsR1 neurons. A) GFP expression induced by Cre-inducible AAV-GFP injected into the VTA of *NtsR1^{Cre};GFP* transgenic mice. Yellow arrows indicate NtsR1 neurons with reduced GFP expression that do not express endogenous L10-GFP and colocalize with TH. **B)** Endogenous L10-GFP expression in the VTA of uninjected *NtsR1^{Cre};GFP* mice reveals fewer labeled neurons compared to AAV-GFP-injected mice of the same genotype. Scale bar represents 100 µm. *ip=interpeduncular nucleus, ml=medial lemniscus*.

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Chapter 5. A Central Neurotensin Circuit That Coordinates Weight Loss Behaviors

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This chapter is a modified version of a manuscript under review

5.1 Abstract

The central mechanism by which neurotensin (Nts) potentiates weight loss has remained elusive. We leveraged chemogenetics to reveal that Nts-expressing neurons of the lateral hypothalamic area (LHA) promote weight loss by increasing volitional activity and restraining food intake. Intriguingly, these dual weight loss behaviors are mediated by distinct signaling pathways: Nts action via NtsR1 is required for the anorectic effect of the LHA Nts circuit, but not for regulation of locomotor or drinking behavior. Furthermore, although LHA Nts neurons cannot reduce intake of freely available obesogenic foods, they effectively restrain motivated feeding in hungry, weight-restricted animals. LHA Nts neurons are thus vital mediators of central Nts action, particularly in the face of negative energy balance. Enhanced action via the LHA Nts circuit may be useful to suppress the increased appetite that occurs after lifestyle-mediated weight and prevent weight regain.

5.2 Introduction

Obesity is a formidable health concern yet diet and exercise remain the only effective non-surgical treatments to support weight loss[1]. Individuals who lose weight via lifestyle

modification experience compensatory increases in appetite and diminished metabolic rate, and as a result, most regain weight[2,3]. Understanding how the brain coordinates feeding and energy expenditure is therefore crucial to identify strategies that support sustained weight loss.

Dopamine (DA) released from the ventral tegmental area (VTA) and substantia nigra (SN) is essential for feeding behavior, volitional physical activity, and the maintenance of body weight[4]. In particular, VTA DA released to the nucleus accumbens (NA) modifies food seeking and "wanting" of palatable foods that can override homeostatic need, leading to overconsumption of calorie-rich food and weight gain[5-7]. Indeed, DA signaling is altered in obesity[8,9] but broad pharmacologic manipulation of DA action (with effects in the NA and other sites of DA release) provides only modest weight loss[1]. Selective modulation of mesolimbic DA signaling may be useful to modulate feeding and weight without off-target effects. One possible candidate to modify mesolimbic DA action and body weight is the neuropeptide, neurotensin (Nts). Direct application of Nts to the VTA suppresses food intake, increases locomotor activity, and increases DA release to the NA[10-12]. Nts signals via Nts receptors 1 and 2 (NtsR1 and NtsR2), and NtsR1 in particular is expressed on a subset of VTA DA neurons and has been implicated in Nts-mediated suppression of feeding[13-15]. Although Nts expression is reduced in the brain of obese animals[16], treatment with NtsR1 agonists reduces their food intake and promotes weight loss[13]. Thus, Nts is a putative anorectic signal, but the neural circuits by which Nts engages the mesolimbic DA system to coordinate energy balance remain to be elucidated.

An important source of central Nts originates from the lateral hypothalamic area (LHA), a crucial hub for coordinating peripheral energy cues with DA-mediated motivated behaviors to regulate body weight[14,17]. The numerous Nts-expressing neurons in the LHA are distinct from orexigenic neurons expressing melanin-concentrating hormone and orexin/hypocretin, project to the VTA and are activated by signals that suppress feeding such as the appetite-suppressing

hormone, leptin[18]. Indeed, loss of leptin signaling via LHA Nts neurons increases adiposity through diminished volitional physical activity, and disrupts coordination between peripheral energy status and motivated feeding behavior[18,19]. By contrast, experimental activation of LHA Nts neurons increases Nts release in the VTA, promotes NA DA release via NtsR1, and enhances locomotor activity[20]. We therefore hypothesized that LHA Nts neurons are physiological mediators of Nts-mediated weight loss behaviors. Using a chemogenetic strategy (DREADDs) to selectively activate LHA Nts neurons in the presence and absence of NtsR1, we reveal an important role for these neurons, and specifically for Nts action via NtsR1 in regulating body weight.

5.3 Results

To experimentally activate LHA Nts neurons and simultaneously determine the requirement for Nts signaling via NtsR1, we crossed *Nts*^{Cre} mice to a NtsR1 knockout line, generating *Nts*^{Cre} mice that have either intact or absent NtsR1 signaling (*Nts*^{Cre}*;*++ and *Nts*^{Cre}*;NtsR1*^{KOKO} mice); these mice are henceforth referred to as *WT* and *NtsR1KO*. Adult *WT* and *NtsR1KO* mice were injected with Cre-inducible AAV-hM3Dq-mCherry in the LHA to express excitatory DREADD receptors selectively in LHA Nts neurons (Fig. 27A). Treating mice with clozapine-N-oxide (CNO) induced cFos in mCherry-labeled LHA Nts neurons, verifying that this method activates LHA Nts neurons (Fig. 27B). We confirmed that *WT* and *NtsR1KO* mice have similar amounts of mCherry-labeled Nts cell bodies in the LHA and terminals in the VTA, indicating that developmental deletion of NtsR1 does not structurally disrupt the LHA Nts circuit (Fig. 27 C, D). Additionally, *NtsR1KO* mice lack VTA *Ntsr1,* but they do not exhibit any compensatory increase in the other signaling receptor for Nts, NtsR2, (Fig. 27Ee). Furthermore, although LHA Nts neurons project to the VTA and within the LHA[14,19], the dearth of LHA NtsR1 neurons suggests that Nts signaling is predominantly mediated via the dense population of VTA NtsR1 neurons (Fig. 27F). Together these data confirm an intact LHA Nts projection to the

NtsR1-rich VTA, and that activating LHA Nts neurons in *WT* and *NtsR1KO* mice can reveal the physiological role of Nts action via this circuit

Acute Activation of LHA Nts Neurons Promotes Activity and Suppresses Feeding: We first examined how activation of LHA Nts neurons alters energy balance by analyzing *WT* and *NtsR1KO* mice in metabolic chambers after single injections of VEH or CNO. Activation of LHA Nts neurons robustly increased water intake over 8 hr in *WT* mice and (to a lesser extent) in *NtsR1KO* mice, suggesting that NtsR1 is not essential for LHA Nts-induced drinking (Fig. 28A, B). *WT* and *NtsR1KO* mice also exhibited increased locomotor activity and energy expenditure for several hours after CNO-mediated activation of LHA Nts neurons (Fig. 28C-F). CNO-treated mice were less active during the dark cycle, presumably to compensate for increased light cycle activity, yet the locomotor activity over 24 hr was significantly increased in *WT* but not *NtsR1KO* mice (Fig. 29A-C). Energy expenditure over 24 hr was elevated in both *WT* and *NtsR1KO* mice (Fig. 29D, E, p=0.051 for *NtsR1KO* group), indicating that that activation of LHA Nts neurons enhances caloric usage in an NtsR1-independent manner. Given the increased energy demands induced by activation of LHA Nts neurons, we examined whether *WT* or *NtsR1KO* mice consumed more calories to maintain energy balance. Interestingly, *WT* mice did not increase feeding after CNO injection, when they are also most active (Fig. 28G vs. C), and reduced feeding during the dark cycle resulting in decreased total food intake over 24 hr (Fig. 29F-H). By contrast, *NtsR1KO* mice increased their food intake immediately after CNO injection and minimally reduced it throughout the dark cycle, resulting in no feeding difference over 24 hr compared to VEH treated mice (Fig 28H, Fig 29F-H). While activation of LHA Nts neurons in WT mice suppressed feeding, increased energy expenditure and resulted in weight loss, the *NtsR1KO* mice retained weight due to their compensatory food intake (Fig. 28I). Collectively, these data suggest that activation of LHA Nts neurons promotes locomotor activity and energy

expenditure, and that Nts signaling via NtsR1 is required to concomitantly suppress feeding and promote weight loss.

To verify the requirement for NtsR1 in LHA Nts-induced anorectic behavior, we pre-treated *WT* and *NtsR1KO* mice with the NtsR1 antagonist SR48692 (0.3 mg/kg, *i.p.*), then administered VEH or CNO to activate LHA Nts neurons. The NtsR1 antagonist had no effect on the CNO-induced water intake or locomotor activity of *WT* and *NtsR1KO* mice (Fig. 30A, B, D, E), confirming that LHA Nts neurons promote these behaviors by an NtsR1-indpendent mechanism. By contrast, the LHA Nts-mediated reduction of chow intake over 24 hr was abolished in *WT* mice pre-treated with the NtsR1 antagonist (Fig. 30C). Thus, activation of LHA Nts neurons while NtsR1 signaling was either pharmacologically or genetically disrupted promoted increased feeding during the light cycle, perhaps to compensate for increased energy expenditure (Fig. 31A, B, Fig. 28H). These data suggest that NtsR1 is necessary for LHA Nts neurons to acutely suppress feeding. Since NtsR1 is expressed on VTA DA neurons[14] and activation of LHA Nts neurons promotes DA release[20], we next investigated whether DA signaling is necessary for LHA Nts-induced weight-loss behaviors. Pre-treatment with the D1 receptor antagonist SCH23390 (0.1 mg/kg *i.p.*) prior to activating LHA Nts neurons did not block drinking behavior in *WT* mice (Fig. 30G). Drinking was generally blunted in *NtsR1KO* mice during this experiment as they acclimated to the lixits in the metabolic cages, but the D1 receptor antagonist did not significantly block their water intake compared to PBS treatment (Fig. 30J). Together these data suggest that DA signaling via D1R is not required LHA Nts-induced water intake. Consistent with previous work[20], D1 receptor antagonism blunted LHA Nts-induced locomotor activity in *WT* and *NtsR1KO* mice (Fig. 30H, K, p=0.06 for *NtsR1KO* mice). D1R blockade also abolished the ability of LHA Nts neurons to suppress feeding in *WT* mice, similar to the effect of disrupting NtsR1 signaling (Fig. 30I, Fig. 31C, D). D1R blockade did not alter LHA Nts-induced hypophagia in *NtsR1KO* mice, as lack of NtsR1 on VTA DA

neurons likely impedes the ability of LHA Nts neurons to induce DA release to the NA. In sum, these data indicate that NtsR1 and D1 signaling are required for LHA Nts neurons to acutely restrain food intake.

LHA Nts Action Promotes Sustained Weight Loss: To determine if LHA Nts action could sustain weight loss, we activated the LHA Nts neurons of *WT* mice in their home cages for 5 consecutive days. Similar to our previous observations, activating LHA Nts neurons non-significantly diminished chow intake on day 1 (Fig. 32A, p=0.14), but did not alter feeding on days 2-5. The increased error of hand-weighing chow from home cages, compared to the previous automated measurements from metabolic cages likely reduced our ability to detect significant feeding differences on day 1. Nonetheless, *WT* mice lost weight after the first injection of CNO and maintained that reduced body weight throughout the study (Fig. 32B). Given that acute activation of LHA Nts neurons promoted weight loss via both suppressing feeding and increasing physical activity and energy expenditure, we measured the physical activity of mice in locomotor chambers for 1 hr after their final VEH or CNO treatment on day 5. We observed a strong trend towards increased activity in CNO-treated mice (Fig. 32C, p=0.06), suggesting that chronic activation of LHA Nts neurons maintains elevated locomotor activity and presumably increased energy expenditure. Chronic activation of LHA Nts neurons also sustained vigorous water consumption throughout the study (Fig. 32D). Importantly, CNO treatment had no effect on *WT* or *NtsR1KO* mice expressing Cre-inducible channel rhodopsin (ChR) in LHA Nts neurons instead of hM3Dq-mCherry, confirming that our results are not due to off-target effects of CNO (Fig. 33). Collectively, these data demonstrate that repeated activation of LHA Nts neurons supports sustained weight loss.

Role of LHA Nts Neurons in Palatable Food Intake: Next we investigated whether activating LHA Nts neurons could support weight loss in mice made obese by free access to a

palatable high fat (45%), high sucrose diet (HF diet). Obese *WT* mice expressing hM3Dq-mCherry in LHA Nts neurons were given daily treatments of VEH or CNO while assessed in metabolic chambers. As in normal weight mice, chronic activation of LHA Nts neurons promoted locomotor activity immediately after CNO injection, followed by a compensatory decrease in activity during the dark cycle (Fig. 34A, B). Despite a trend toward increased total locomotor activity over 5 days in CNO-treated mice ($p=0.07$, data not shown), chronic activation of LHA Nts neurons did not significantly increase energy expenditure in obese mice as observed in lean animals (Fig. 34C vs. Fig. 28E, F, Fig. 29D, E). Whereas activating LHA Nts neurons suppressed feeding in normal weight mice, activation in obese mice increased intake of the palatable HF diet during the light cycle with no subsequent suppression of feeding during the dark phase (Fig. 34D, E). As a result, CNO-treated mice maintained their obesity while VEH-treated controls slightly lost weight during the experiment due to handling stress (Fig. 34F). Thus, although activation of LHA Nts neurons promoted locomotor activity in obese mice it was insufficient to offset the consumption of freely available palatable food necessary to support weight loss.

Prolonged obesity disrupts DA signaling[8,9], hence activation of LHA Nts neurons in profoundly obese mice may not be able to appropriately modify mesolimbic DA signaling to promote weight loss behaviors. We therefore tested whether LHA Nts neurons could prevent normal weight mice from HF diet-induced weight gain. Lean chow-fed *WT* mice were switched to *ad lib* palatable HF diet followed by daily VEH or CNO treatments to activate LHA Nts neurons. Similar to obese mice, activation of LHA Nts neurons caused lean *WT* mice to consume significantly more palatable HF diet than VEH-treated controls (Fig. 35A, B). Although activation of LHA Nts neurons also increased locomotor activity (Fig. 35C, measured on day 5), any concomitant increase in energy expenditure was counteracted by increased food intake,

leading to weight gain (Fig. 35D). Thus, while activation of LHA Nts neurons suppresses chow intake, it cannot restrain consumption of freely accessible palatable foods that promote obesity.

LHA Nts Neurons Suppress Motivated Feeding: VTA DA signaling modifies the motivation to obtain food rewards (e.g. "wanting") but their hedonic value ("liking") is mediated via separate systems[7]. Thus, LHA Nts neurons engaging the VTA are not anatomically positioned to modify palatability-driven intake of freely accessible food, consistent with our findings (Figs. 34 and 35). We reasoned, however, that LHA Nts neurons might suppress motivated feeding via Nts-signaling to VTA NtsR1 neurons. We therefore asked if activating LHA Nts neurons in fasted *WT* and *NtsR1KO* mice could blunt their motivation to eat once food was restored[21]. CNO-mediated activation of LHA Nts neurons restrained re-feeding, which persisted over 24 hr in *WT* mice (Fig. 36A, B, p=0.10 for *NtsR1KO*s at 24 hrs) and resulted in less weight regain compared to VEH-treated controls (Fig. 36C). These data demonstrate that LHA Nts neurons can suppress chow feeding and subsequent weight gain even under conditions that increase the drive to feed, such as fasting-induced weight loss[22].

To determine if activation of LHA Nts neurons suppresses the motivation to obtain palatable food, *WT* and *NtsR1KO* mice were trained to nose-poke (e.g. work) for sucrose pellets, and both groups learned the task equally well (Fig. 37A-D). We then examined whether VEH treatment or CNO-mediated activation of LHA Nts neurons alters the progressive ratio (PR) breakpoint for sucrose, a measure of the motivation to obtain rewards that is dependent on mesolimbic DA signaling[23]. Activation of LHA Nts neurons prior to testing did not alter PR breakpoints in *ad lib* fed (Sated) *WT* or *NtsR1KO* mice (Fig. 36D). Thus, activation of LHA Nts neurons in an energy-replete context does not suppress freely accessible (Fig. 35) or operant-reinforced intake of palatable food. By contrast, activation of LHA Nts neurons after overnight fasting suppressed sucrose self-administration in *WT* but not *NtsR1KO* mice (Fig. 36D),

suggesting that LHA Nts action via NtsR1 reduces motivated sucrose “wanting” in the face of physiologic energy deficit. Importantly, the increased locomotor activity of mice upon activation of LHA Nts neurons did not impair their ability to obtain sucrose, as magazine entries were unaffected by CNO treatment (Fig. 37E). Together, these data provide evidence that enhanced LHA Nts neuronal activity counteracts the drive to consume palatable food in an energy-restricted state[22,24], similar to what occurs with diet-induced weight loss.

Aversive stimuli or stress[25,26] also suppress feeding, so we investigated if activation of LHA Nts neurons promotes these conditions. First we determined whether *WT* and *NtsR1KO* mice exhibited any aversion for chambers paired with either VEH or CNO. *WT* mice demonstrated neither aversion nor preference for the CNO-paired chamber. By contrast, *NtsR1KO* mice significantly preferred the chamber associated with CNO-mediated activation of LHA Nts neurons (Fig. 38A), but CNO failed to modify place preference in mice expressing ChR in LHA Nts neurons instead of hM3Dq-mCherry (Fig. 38B). Together, these data suggest that intact LHA Nts signaling via NtsR1 does not suppress feeding due to aversion, but loss of action via NtsR1 may underlie the reward-induced feeding observed in *NtsR1KO* and NtsR1-antagonist treated mice. Furthermore, activation of LHA Nts neurons did not increase anxiety-like behavior measured via elevated plus maze (EPM), thus this circuit does not mediate stress-induced suppression of feeding (Fig. 38C, D). Lastly, because we anecdotally observed CNO-induced behaviors such as gnawing, grooming, and digging, we examined if these were directed at procuring food or toward any object, in this case a nestlet placed in the cage. Activation of LHA Nts neurons significantly increased nestlet-shredding in both *WT* and *NtsR1KO* mice (Fig. 38E, F), consistent with a role for increased DA signaling in mediating arousal behavior[27]. Collectively, these data indicate that LHA Nts-mediated suppression of food intake is not confounded by aversion or anxiety, and is associated with locomotor behaviors directed toward non-food objects.

5.4 Discussion

We demonstrate that the LHA Nts circuit increases volitional activity and energy expenditure while suppressing food intake, and hence promotes dual behaviors to support weight loss. Intriguingly LHA Nts neurons modulate these behaviors via distinct mechanisms: Nts-mediated signaling via NtsR1 is required for the anorectic effects of this circuit but the locomotor behavior occurs via an NtsR1-independent mechanism. Although LHA Nts neurons cannot suppress intake of freely accessible palatable foods that promote weight gain, they do restrain feeding in fasted, weight-reduced mice that are highly motivated to eat. Taken together, these data suggest that activation of the LHA Nts circuit might be useful to maintain weight loss in the face of negative energy balance. For example, restraining the increased appetitive drive that occurs after an initial bout of weight loss may prevent weight regain and support further weight loss via continued diet and exercise.

Previous work implicated a role for central Nts signaling in regulating feeding and locomotor activity via modifying DA signaling[10,11,13] yet the specific neural circuits mediating Nts action remained unclear. Similar to the effects of pharmacologic Nts or NtsR1 agonists in the VTA[10-12,28], we demonstrate that activation of LHA Nts neurons that project to the VTA increased physical activity while suppressing food intake in energy-replete mice. However, because LHA Nts-induced locomotor activity did not depend on intact NtsR1, and was only mildly blunted by D1R antagonism, it is likely controlled by a non-Nts signal released from LHA Nts neurons. By contrast, both NtsR1 and D1R antagonism abolished LHA Nts-induced suppression of feeding, implying obligate, overlapping roles for Nts and DA in this process. Given that LHA Nts neurons directly engage VTA NtsR1 neurons to promote DA release[14,20], our data suggest that induction of DA signaling via this circuit functionally restrains feeding to potentiate weight loss. While DA release to the NA is most commonly linked to increased feeding rather than suppression, DA signaling is also necessary for the anorectic and weight-reducing effects of some medications[29].

Thus, discrete DA circuits may differentially modify feeding, and LHA Nts-induced NtsR1 signaling may be a specific pathway to engage mesolimbic DA circuits that suppress appetite.

LHA Nts neurons do not provide an absolute brake on feeding, as their activation only suppressed *ad lib* intake of chow but not HF diet. A possible explanation for this discrepancy is that the palatability and/or caloric value of HF diet overrides the ability of LHA Nts neurons to restrain intake. Mechanistically this may signify that LHA Nts neurons do not modify the opioid signaling systems that encode food "liking" that can drive overconsumption[7]. Intake of sucrose or fat increases DA release independently of taste, and this post-ingestive DA signaling is important for coordinating caloric intake with energy demands[45,46]. Thus, it is possible that activating LHA Nts modifies downstream mesolimbic DA circuits in a way that renders them incapable of sensing post-ingestive cues, causing overconsumption of calorie-dense HF diet, whereas post-ingestive DA signaling may play less of a role during intake of less palatable chow. DA signaling also modifies the motivation to work for food rewards[7] and hence may be important for coordinating the motivation to consume food necessary for survival. For example, the changes in circulating ghrelin and leptin during food-deprivation modulate VTA DA signaling to increase the motivation to eat and restore energy balance[30-32]. Anorectic signals thus more profoundly diminish feeding in fasted animals with an elevated drive to eat, similar to the effect of exogenous leptin treatment, which reduces food intake more effectively in fasted state[21,22]. Similarly, LHA Nts neurons robustly suppress fasting-induced chow intake and weight regain, and may in fact contribute to leptin-mediated suppression of feeding[19]. Interestingly, activation of LHA Nts neurons only suppressed DA-dependent operant responding for palatable food in fasted *WT* mice, but not sated mice. Since feeding suppression is more easily detected when levels of food "wanting" are high (as during fasting), our finding in consistent with the possibility that LHA Nts neurons suppress feeding by reducing the "wanting" of food. The failure of LHA

Nts neurons to suppress operant palatable intake in the absence of NtsR1, however, supports an essential role for Nts action via NtsR1 in limiting both *ad lib* and motivated feeding.

Some LHA Nts-mediated behaviors, including water intake and locomotor activity were not different between *WT* and *NtsR1KO* mice, suggesting that they occur via NtsR1-indpendent mechanisms. Nts may still contribute to these behaviors via engaging NtsR2, which is predominantly expressed by astrocytes in the VTA (Woodworth *et al.*, unpublished). Glia can regulate neuronal signaling[33], so the possibility of LHA Nts signaling acting through NtsR2 to alter behavior deserves consideration in the future. Alternately, signals other than Nts that are released from LHA Nts neurons might mediate drinking and locomotor activity. For example, at least some LHA Nts neurons contain GABA[34], which is presumably co-released with Nts upon DREADD-induced activation. Similar to LHA Nts action, optogenetic activation of LHA GABA neurons increases NA DA release[35], water intake and non-appetitive gnawing[36,37]. Conversely, optogenetic or pharmacogenetic activation of LHA GABA neurons increases feeding and either does not affect or suppresses locomotor activity[25,36,37], whereas LHA Nts activation suppresses chow feeding and increases locomotor activity. Furthermore, LHA GABA neurons that project to the VTA promote conditioned reward[35] whereas LHA Nts neurons only induce such reward in the absence of NtsR1. These discrepancies suggest that LHA Nts neurons do not fully overlap with the population of LHA GABA neurons, and that perhaps only a subset of LHA Nts neurons are also GABAergic. Within this putative subset of LHA Nts-GABA neurons, the loss of Nts signaling via NtsR1 might bias GABAergic actions in the VTA, and could explain our observation that activation of LHA Nts neurons in *NtsR1KO* mice causes conditioned place preference similar to that induced by the activation of LHA GABA neurons. Similarly, hypothalamic Nts is reduced in obesity, and this might also favor GABAergic signaling that potentiates feeding and weight gain, as observed in rodents with experimentally activated LHA GABA neurons[25,36,37]. Alternately, some LHA Nts neurons may co-express glutamate[38] and the phenotype of LHA Nts

activation partially overlaps with activation of all LHA glutamatergic neurons as well. In particular, LHA glutamatergic neurons suppress feeding in food-restricted animals[39], similar to LHA Nts neurons. By contrast, while LHA Nts activation is neither rewarding nor aversive, LHA glutamatergic stimulation produces aversion[39,40], which again supports the idea that Nts is co-expressed on some, but not all LHA glutamatergic neurons. Collectively, we hypothesize that Nts is co-expressed on subsets of glutamatergic and GABAergic LHA neurons, and stimulation of the LHA Nts population as a whole promotes a repertoire of beneficial behaviors that may promote weight loss.

LHA Nts neurons may also differentially control locomotor, drinking and feeding behaviors is via distinct projections. Since LHA Nts neurons robustly project to the VTA, where NtsR1 is expressed almost exclusively on DA neurons, the NtsR1-dependent suppression of feeding is likely mediated via LHA Nts inputs to the VTA. Determining whether VTA-projecting LHA Nts neurons co-release glutamate, GABA, or a combination of both may provide insight into the control of non-feeding behaviors. For instance, LHA Nts neurons might co-release GABA onto VTA GABA interneurons, thereby disinhibiting DA neurons and promoting DA signaling[35]. LHA Nts neurons also project locally to inhibit neighboring orexin (OX) neurons[19,41], which could decrease food intake, especially in a food-restricted state. The mechanism of this, however, is likely independent of NtsR1 signaling given the lack of NtsR1 expression in the LHA, and could explain why both *WT* and *NtsR1KO* mice have suppressed food intake with LHA Nts activity in a fasted state. Our data thus reveal that LHA Nts neurons are important mediators of central anorectic Nts action via NtsR1. Future work will be key to define the additional signaling mechanisms by which LHA Nts neurons support weight loss behaviors.

5.5 Methods

Reagents: CNO was obtained from the NIH as part of the Rapid Access to Investigative Drug Program funded by the NINDS. 40x CNO stock solutions were made by diluting with PBS/10% beta-cyclodextrin (Sigma). VEH was PBS. SR48690 was purchased from Sigma and 20x stock solutions were made in 1% TWEEN. SCH23390 was also purchased from Sigma and 50x stock solutions were made in PBS. All stock solutions were aliquoted and stored at -20 until use.

Animals: Mice were bred and housed in a 12h light/12h dark cycle and cared for by Campus Animal Resources (CAR) at Michigan State University. Animals had *ad lib* access to chow (Teklad 7913) and water unless otherwise noted. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Generation of NtsR1$^{IRES\text{-}Cre}$ Knock-In Mice: An *NtsR1$^{IRES\text{-}Cre}$* targeting vector was generated by inserting an IRES-Cre sequence between the stop codon and the polyadenylation site of the sequence encoding the 3' end of the mouse *Ntsr1* gene, with an *frt*-flanked Neo cassette inserted upstream of the IRES-Cre. The linearized targeting vector was electroporated into mouse R1 embryonic stem (ES) cells (129sv background) and cells were selected with G418. DNA from ES cell clones was analyzed via qPCR for loss of homozygosity using Taqman primer and probes for the genomic *Ntsr1* insertion site (Forward: TCT GAT GTT GGA CTT GGG TTC, Reverse: TCT GAT GTT GGA CTT GGG TTC, Probe: TCT GAT GTT GGA CTT GGG TTC). *NGF* was used as a copy number control[42]. Putative positive ES clones were expanded, confirmed for homologous recombination by Southern blot and injected into mouse C57BL/6 blastocysts to generate chimeras. Chimeric males were mated with C57BL/6 females (Jackson Laboratory), and germline transmission was determined initially via progeny coat color, then confirmed via conventional PCR for IRES-Cre.

Breeding and Genotyping: To generate breeders, heterozygous *Nts^{cre}* mice[19] (Jackson stock #017525) were mated to *NtsR1^{KO+}* (Jackson stock #005826) mice, and progeny with the genotypes *Nts^{Cre};NtsR1^{KO+}* and *++; NtsR1^{KO+}* were subsequently mated to generate *Nts^{Cre};++* and *Nts^{Cre};NtsR1^{KOKO}* study animals. To maximize animal usage, study animals also arose from the following crosses: *Nts^{Cre}:++* X WT or *Nts^{Cre};NtsR1^{KOKO}* X *++;NtsR1^{KOKO}.* To visualize NtsR1 neurons, *NtsR1$^{IRES\text{-}Cre}$* mice were crossed to homozygous *Rosa26$^{EGFP\text{-}L10a}$* mice and progeny heterozygous for both alleles were studied. Genotyping was performed with standard PCR using the following primer sequences: *Nts^{cre}:* common forward: 5' ATA GGC TGC TGA ACC AGG AAC, reverse: 5' CCA AAA GAC GGC AAT ATG GT and WT reverse: 5' CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26$^{EGFP\text{-}L10a}$* : mutant forward: 5' TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5' GAG GGG AGT GTT GCA ATA CC and common reverse: 5' CAG ATG ACT ACC TAT CCT CCC. *NtsR1^{KO}:* Forward: CTC TAA TGT GCC ACA GCT CAG AGA G, common: CAG CAA CCT GGA CGT GAA CAC TGA C, Reverse: CCA AGC GGC TTC GGC CAG TAA CGT T *NtsR1$^{IRES\text{-}Cre}$:* Forward: GGA CGT GGT TTT CCT TTG AA, Reverse: AGG CAA ATT TTG GTG TACG G.

Surgery: At 8-12 weeks of age, male *Nts^{Cre};++* and *Nts^{Cre}:NtsR1^{KOKO}* mice received a pre-surgical injection of carprofen (5mg/kg *s.c.*) and were anesthetized with 3-4% isoflurane/O_2 in an induction chamber before being placed in a stereotaxic frame (Kopf). Under 1-2% isoflurane, access holes were drilled in the skull allowing a guide cannula with stylet (PlasticsOne) to be lowered into the brain target area. Mice were injected bilaterally with either 300 uL of AAV-hM3Dq-mCherry or AAV-ChR-mCherry (UNC vector core) in the LHA (AP: -1.34, ML: -1.05, DV: -5.2). After 5 min to allow for virus absorption, the injector and cannula were removed from the skull and the incision was closed using Vet Bond. Analysis began 1-2 weeks after recovery. Mice were only included in the final study data if injections were confined to the

LHA on both sides. Approximately 90% of animals included in the study were bilaterally targeted; however in 10% of cases, animals with robust unilateral targeting were included in the study if CNO injection induced >1mL of water consumption, as analysis of several cohorts revealed this as a reliable indicator of LHA Nts targeting. To visualize NtsR1 neurons, adult male and female *NtsR1^{Cre};GFP* mice were bilaterally injected with 1uL FlpO adenovirus (Vector Biolabs) into the lateral ventricles (A/P: -0.22, M/L: +/- 1.0, D/V: -2.0). Mice were perfused 10 days after surgery to permit sufficient time for FlpO-mediated excision of the *frt*-flanked Neo cassette and GFP expression.

Metabolic Analysis: At 1-2 weeks post-surgery, mice were placed in TSE cages for metabolic phenotyping (PhenoMaster, TSE Systems). After 24-48 hours of acclimation, mice were *i.p.* injected 1-2 hr after the onset of the light cycle with PBS, SR48692 (0.3 mg/kg), or SCH23390 (0.1mg/kg), followed by VEH or CNO (0.3 mg/kg) 30 min later. Mice were given a 24 hr washout between PBS and antagonist injections. They were continuously monitored for food and water intake, locomotor activity, and energy expenditure. Ambient temperature was maintained at 20-23˚C and the airflow rate through the chambers was adjusted to maintain an oxygen differential around 0.3% at resting conditions. Metabolic parameters including VO_2, respiratory exchange ratio, and energy expenditure were assessed via indirect calorimetry by comparing O_2 and CO_2 concentrations relative to a reference cage.

Gene Expression: Male 12-16wk old *WT* (++/++) and *NtsR1KO* mice (*NtsR1^{KOKO}*) were deeply anesthetized with sodium pentobarbital and tissue from the VTA was microdissected and immediately snap frozen on dry ice and stored at -80˚C (n=10-11 per group). RNA was extracted using Trizol (Invitrogen) and 200 ng samples were converted to cDNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Sample cDNAs were analyzed in triplicate via quantitative RT-PCR for gene expression using TaqMan reagents and

an ABI 7500 (Applied Biosystems). With *GAPDH* expression as an internal control, relative mRNA expression values are calculated by the $2^{-\Delta\Delta Ct}$ method. *Ntsr1* expression levels in 10 of 11 *NtsR1KO* mice were undetectable and fold change was reported as 0 for those samples.

Chronic Activation in Lean Mice: Chow-Experiments: Mice were injected with VEH or CNO between 8-9AM once daily for five consecutive days in home cages while fed *ad lib* standard chow (Harlan Teklad 7913). Food, water, and body weight were weighed prior to the morning injection and again between 5-6pm. On day 5, locomotor activity was assessed in CPP chambers described below. Animals were randomly assigned to one side of the chamber and were allowed to acclimate for 30 min. Following acclimation, they received VEH or CNO and locomotor activity was measured by laser beam breaks for 1 hr. The study was performed using a cross-over design such that each animal received both VEH and CNO over the course of two separate experiments, with 24 hr of rest between studies.

HF-Diet Experiments: As above, mice were injected once daily with VEH or CNO between 8-9AM in home cages with ad lib access to high fat, high sugar diet (D12451, Research Diets). Mice received 24 hr of access to HF diet prior to the study. On day 5, locomotor activity was assessed as described above, and injections were given for 10 consecutive days to allow time for weight gain. These experiments were also performed using a cross-over design, however mice were given a two-week washout period in between studies with access only to standard chow between experiments.

Activation of LHA Nts Circuit in Obese Mice: Four week old male Nts^{Cre};++ mice were weaned onto 45% high fat, high sucrose diet (HF diet, D12451, Research Diets) to induce obesity. After 6-8 months of HF diet consumption, AAV-hM3Dq-mCherry was stereotaxically delivered in the LHA bilaterally as described above. Following a 2 wk recovery period, obese mice were then analyzed in TSE metabolic cages as described above. Following two days of

acclimation, mice were divided into separate treatment groups and injected with VEH or CNO for 5 consecutive days in TSE cages at 8AM each morning.

Operant Testing: Based on methods by Fulton *et al*.[21], mice were food restricted to 90% of their body weight and trained on a fixed ratio-1 (FR1) schedule until they achieved 75% response accuracy with ≥20 rewards earned on 3 consecutive days. Training sessions were terminated after 1 hr or when the animal had earned 50 rewards. Mice achieving these criteria were then switched to *ad lib* food and trained on an FR5 schedule for 3 consecutive days. On test days, mice were subject to a progressive ratio (PR) schedule were $PR=[5e^{(R*0.2)}]-5$ with R=number food rewards earned+1. Thus, the number of correct responses needed to earn a sucrose reward increases as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 *etc*. The PR breakpoint was recorded as the highest ratio completed for each 1 hr test session. Mice were tested until they achieved stable PR which was defined as <10% variation in rewards earned over 3 consecutive sessions.

Conditioned Place Preference (CPP): CPP boxes (San Diego Instruments) consisted of a box divided into two chambers with different visual and tactile cues (gray wall and smooth floor or striped wall and rough floor) separated by a small center chamber. *Day 1 (Pre-test):* Mice were allowed to roam freely between chambers for 15 min. After pre-test data were collected, an unbiased, counterbalanced strategy was then used to assign which chamber was paired with either VEH or CNO injection, such that approximately half the mice received CNO pairing in the preferred side and half received CNO in the non-preferred side. *Days 2-5 (conditioning):* Each morning, mice received an injection of VEH and were immediately placed in the VEH-paired side for 30 min. After the session, mice were returned to their housing environment to facilitate memory consolidation and 4 hours later, they were conditioned with CNO for 30 minutes in the opposite chamber. *Day 6 (post-test):* Mice were again allowed two

roam freely amongst both chambers for 15 minutes. Time-spent and locomotor activity in each side of the box was assessed by laser beam breaks and data were gathered using the manufacturer's software. One *NtsR1KO* animal was excluded for displaying strong side-preference in the pre-test (>75% of time spent on that side).

Nestlet-Shredding: Immediately after receiving VEH or CNO injection, mice received a white cotton pillow (Ancare) in their home cages, which was weighed prior to placement in the cage. After 90 min, any intact remnants of the original pillow were removed from the cage and allowed to air-dry overnight. The pillow remnants were weighed the following day.

Elevated Plus Maze (EPM): Mice were assessed for anxiety-like behavior using an elevated plus maze (EPM) as previously described[43]. Briefly, the EPM apparatus was custom-built based on plans from ANY-maze (www.anymaze.com, Stoelting Co.) and mice were given free access to the open and closed arms for 5 minutes. Their behavior was recorded using a digital CCD camera and the percentages of time spent in the open and closed arms were analyzed using Topscan automated video tracking software (Clever Sys).

Fasting-Induced Re-feeding: Chow pellets were removed from home cages around 5PM and mice were given a clean cage bottom to prevent feeding of food that may have fallen into the bedding. Mice had *ad lib* access to water during food-deprivation. The following morning between 8AM-9AM, fasted mice were given *i.p.* VEH or CNO and chow pellets were returned to the feeder. Food intake, water intake, and body weight was measured at 1 hr and 24 hr after injection. The study was performed using a cross-over design, such that half the mice received VEH or CNO, and after 3 full days of recovery from fasting, the experiment was repeated with the opposite treatment allowing each animal to serve as its own control.

Immunohistochemistry: Mice were treated with a lethal dose of *i.p.* pentobarbital followed by transcardial perfusion with 10% neutral-buffered formalin (Fisher Scientific). Brains were removed, post-fixed in 10% formalin overnight at 4˚C, dehydrated with 30% sucrose in PBS for 2-3 days, and sectioned into 30 µm slices using a sliding microtome (Leica). Brain sections were then analyzed by immunofluorescence or immunohistochemistry as previously described[14,19]. For characterization of NtsR1 expression, sections from *NtsR1^{Cre};GFP* mice were stained with chicken anti-GFP (1:2000, Abcam) followed by donkey anti-chicken conjugated to AlexaFluor 488 (Jackson ImmunoResearch). For DREADD studies, *WT* and *NtsR1KO* mice were treated with VEH or CNO 90 minutes prior to perfusion, and brain sections were stained for cFos (1:500, goat, Santa Cruz) with secondary detection via DAB (Life Technologies), followed by immunofluorescent detection of dsRed (1:1000, Clontech) as described above. Brains were analyzed using an Olympus BX53 fluorescence microscope outfitted with transmitted light to analyze DAB-labeled tissue, as well as FITC and Texas Red filters. Microscope images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera, and images were analyzed using Photoshop software (Adobe).

Statistics: Student's t-tests and 2-way ANOVA were calculated using Prism 6 (GraphPad). Repeated measures two-way ANOVA with Sidak post-tests was used when each animal was given both VEH and CNO, and when data from the same animals were collected at different time points. For energy expenditure data, analysis of covariance (ANCOVA) was computed in SPSS 22 (IBM). Body weight was analyzed as a covariate to correct for any inherent differences it may have on metabolism[44]. Data were tested for homogeneity of regression, independence of the covariate (body weight), and linearity of regression prior to running the ANCOVA. For all data, *$p<0.05$, **$p<0.01$ and ***$p<0.001$. (#) is used to indicate comparison to starting time line within a given group.

APPENDIX

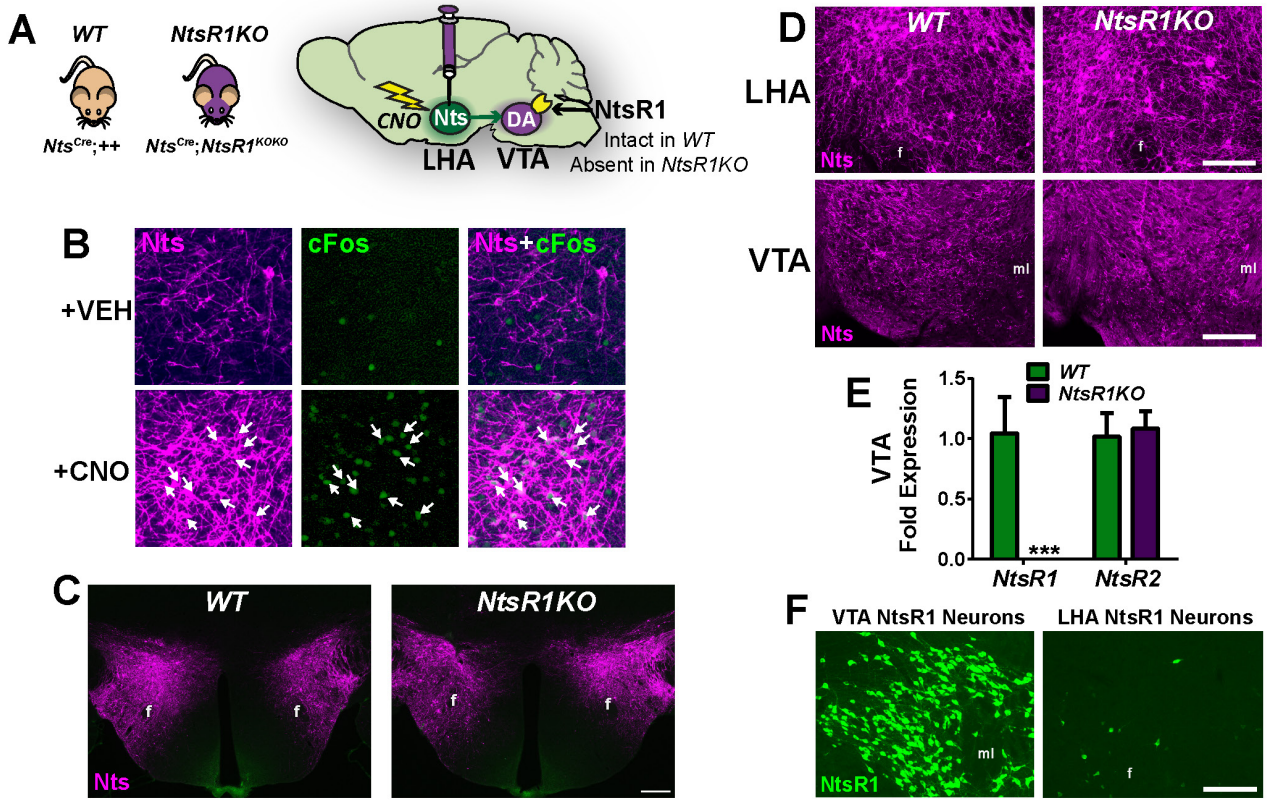

Figure 27. Examination of the LHA Nts→VTA circuit in *WT* and *NtsR1KO* mice. A) *Nts*^{Cre} mice with either intact or developmentally deleted NtsR1 (*WT* or *NtsR1KO* mice) were injected bilaterally in the LHA with cre-inducible AAV-hm3Dq-mCherry, permitting pharmacogenetic activation of LHA Nts neurons by CNO injection. **B)** *i.p* CNO treatment induces cFos expression in LHA Nts neurons (white arrows). **C)** LHA Nts neurons expressing hM3Dq-mCherry in *WT* and *NtsR1KO* mice (scale bar=200μm). **D)** hM3Dq-mCherrry-labeled LHA Nts cell bodies in the LHA and terminals in the VTA of *WT* mice. **E)** *Ntsr1* and *Ntsr2* mRNA expression in the VTA of *WT* and *NtsR1KO* mice (n=10-11 per group). Data were analyzed by unpaired-test for each gene. **F)** NtsR1 expression in *NtsR1*$^{IRES\text{-}Cre}$*;GFP* mice in the LHA and VTA. For **D** and **F**, scale bar=100μM. *f=fornix, ml=medial lemniscus.*

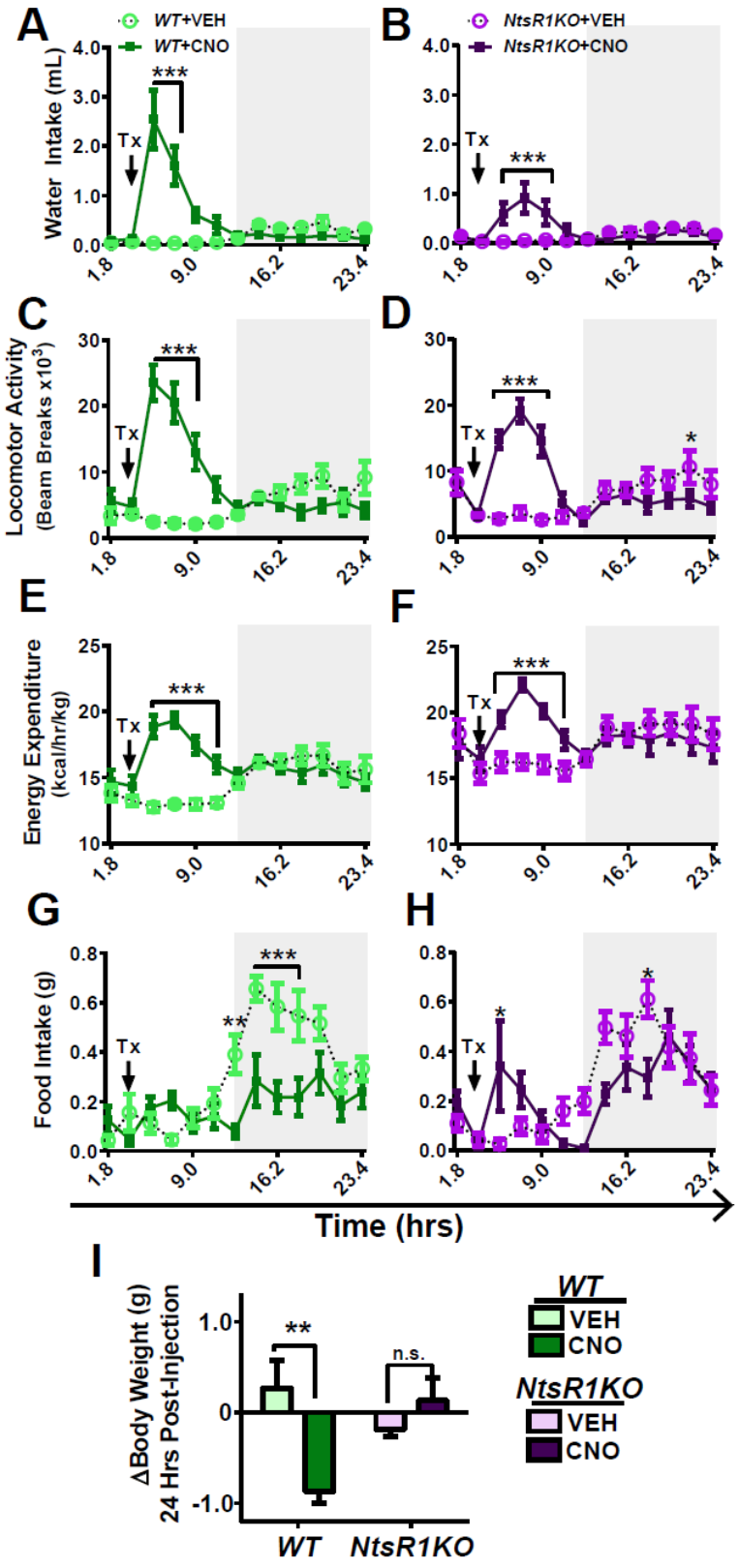

Figure 28. Acute activation of LHA Nts neurons promotes energy expenditure and suppresses feeding.

Figure 28 (*cont'd*). VEH or CNO-treated *WT* and *NtsR1KO* mice were analyzed in TSE metabolic cages to understand how acute LHA Nts neuron activation alters energy balance. In **A-H**, each point represents 108 minutes and gray boxes denote the dark cycle. **A, B)** water intake, **C, D)** locomotor activity, **E, F)** energy expenditure, and **G, H)** feeding in *WT* and *NtsR1KO* mice. **I)** total change in body weight 24 hours post-injection. Data were analyzed by repeated measures two-way ANOVA. *WT* n=10-11; *NtsR1KO* n=9-10.

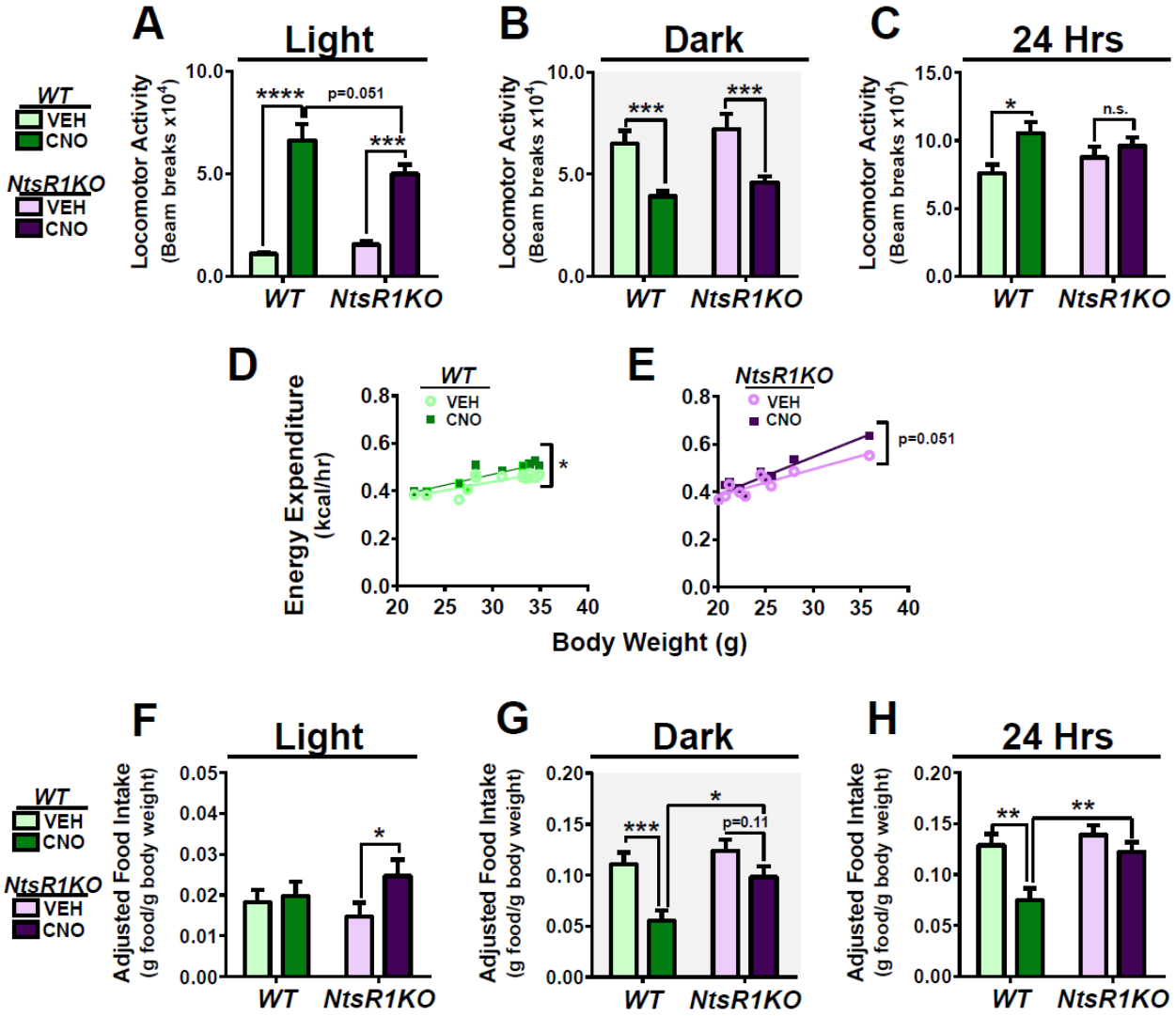

Figure 29. Additional acute metabolic data in *WT* and *NtsR1KO* mice. Light refers to the period 9 hours post VEH or CNO injection while dark refers to data collected during dark cycle (gray background). **A)** Locomotor activity during the light cycle, **B)** dark cycle, and **C)** over 24 hours. **D, E)** Average energy expenditure over 24 hours with VEH or CNO injection. **F)** Weight-adjusted food intake during the light cycle, **G)** dark cycle, and **H)** total over 24 hours. Bar graphs were analyzed by repeated measures two-way ANOVA, while energy expenditure was analyzed by ANCOVA. *WT* n=10-11; *NtsR1KO* n=9-10.

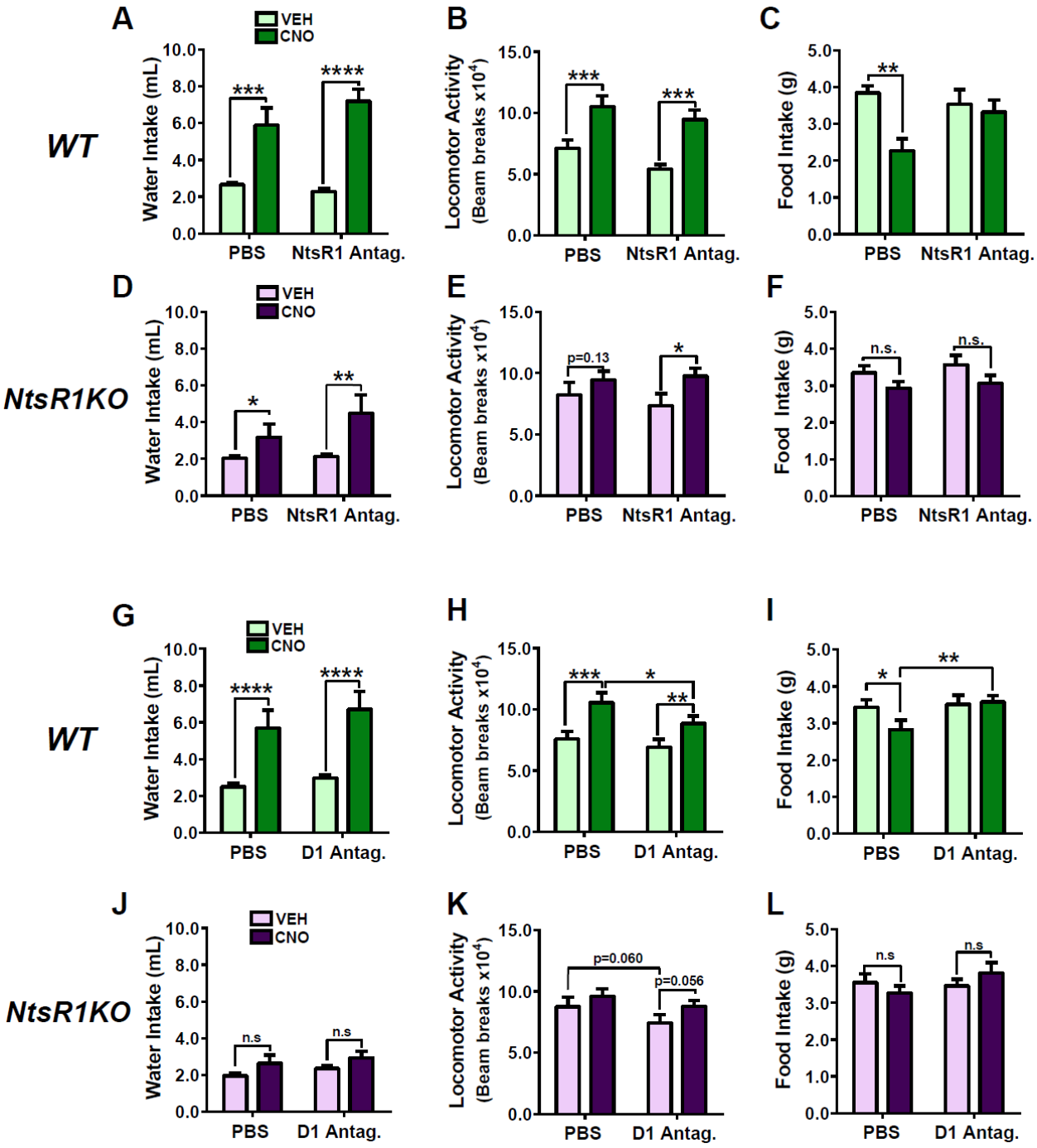

Figure 30. Acute NtsR1 or D1R blockade abolishes LHA Nts-induced suppression of feeding. Mice were pretreated with PBS, SR48692 (NtsR1 antagonist) or SCH23390 (D1R antagonist) 30 min. prior to VEH or CNO injection. Each bar reflects ingestive behavior or locomotor activity over 24 hours post VEH or CNO injection. **A, B, C)** Water intake, locomotor activity and feeding in *WT* mice pretreated with the NtsR1 antagonist. **D, E, F)** Water intake, locomotor activity and feeding in *NtsR1KO* mice pretreated with a NtsR1 antagonist. **G, H, I, J, K, L)** Same parameters in *WT* or *NtsR1KO* mice pretreated with a D1R antagonist (SCH23390). Data were analyzed by repeated measures two-way ANOVA. *WT* n=10-11; *NtsR1KO* n=9-10.

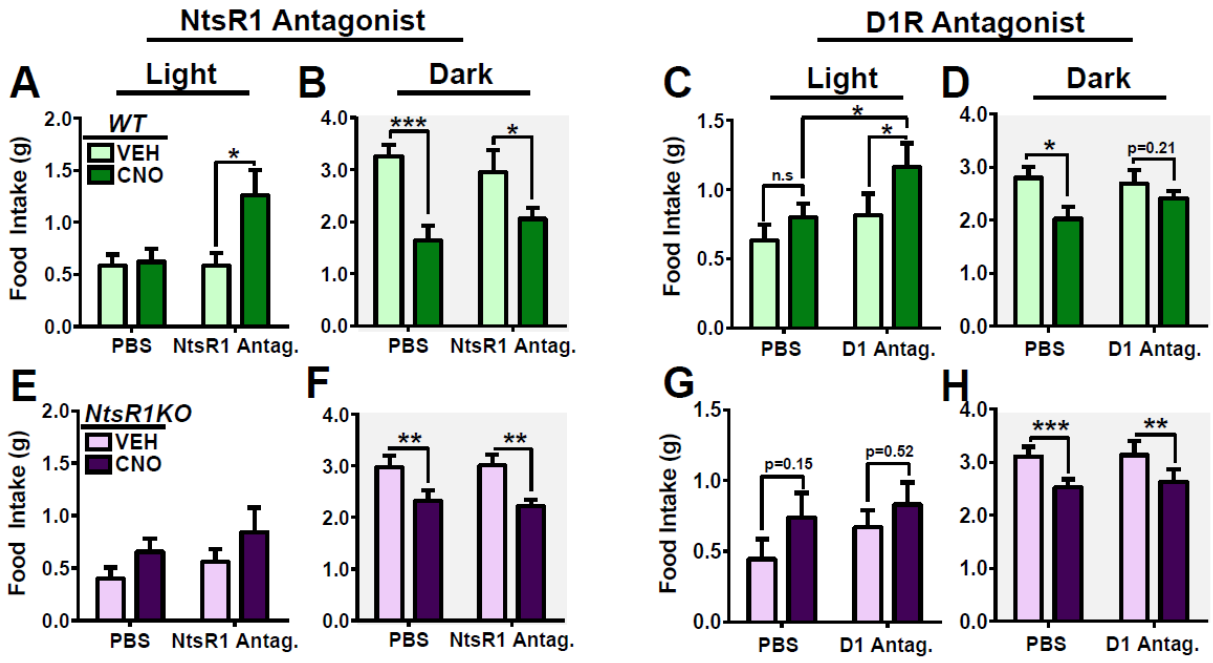

Figure 31. Food intake separated by circadian period with NtsR1 or D1R antagonists. **A, B)** Food intake in *WT* mice during light or dark phases with NtsR1 antagonist pre-treatment or **C, D)** D1 receptor pre-treatment. **E, F)** Food intake in *NtsR1KO* mice during light or dark with NtsR1 antagonist pre-treatment or **G, H)** D1R antagonist pre-treatment. Data were analyzed by repeated measures two-way ANOVA.

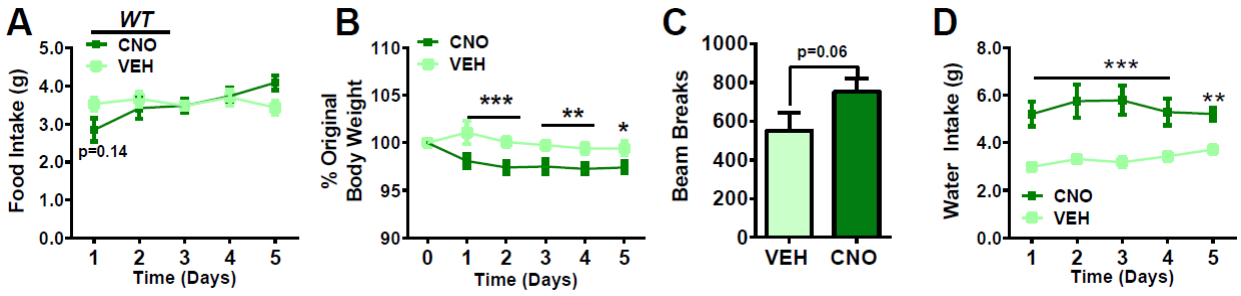

Figure 32. Chronic activation of LHA Nts neurons induces mild weight loss in chow-fed lean mice. *WT* and *NtsR1KO* mice were treated once daily with VEH or CNO for five consecutive days. **A)** chow intake, **B)** body weight, **C)** 1 hr locomotor activity, and **D)** water intake in *WT* mice (n=11). Data were analyzed by repeated-measures two-way ANOVA, except for locomotor activity which was analyzed by paired t-test.

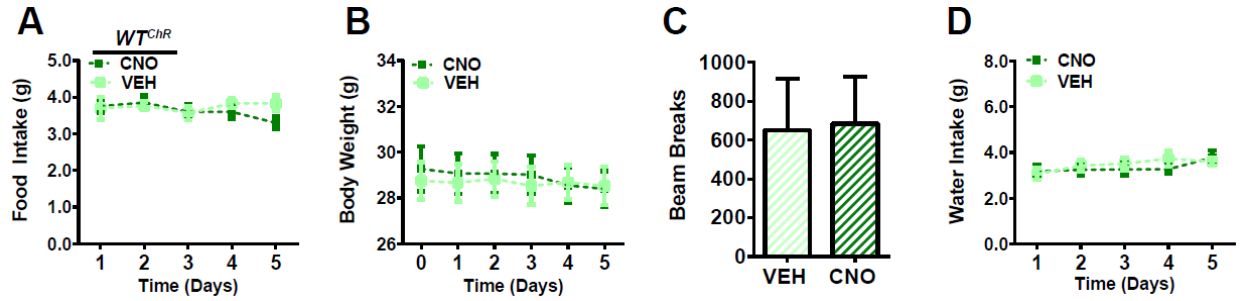

Figure 33. Chronic VEH or CNO injection in *WT*^{ChR} controls. A) Chow intake, **B)** body weight, **C)** 1 hr locomotor activity, and **D)** water intake in *WT*^{ChR} controls (n=6). Data were analyzed by repeated-measures two way ANOVA, except for locomotor activity which was analyzed by paired t-test.

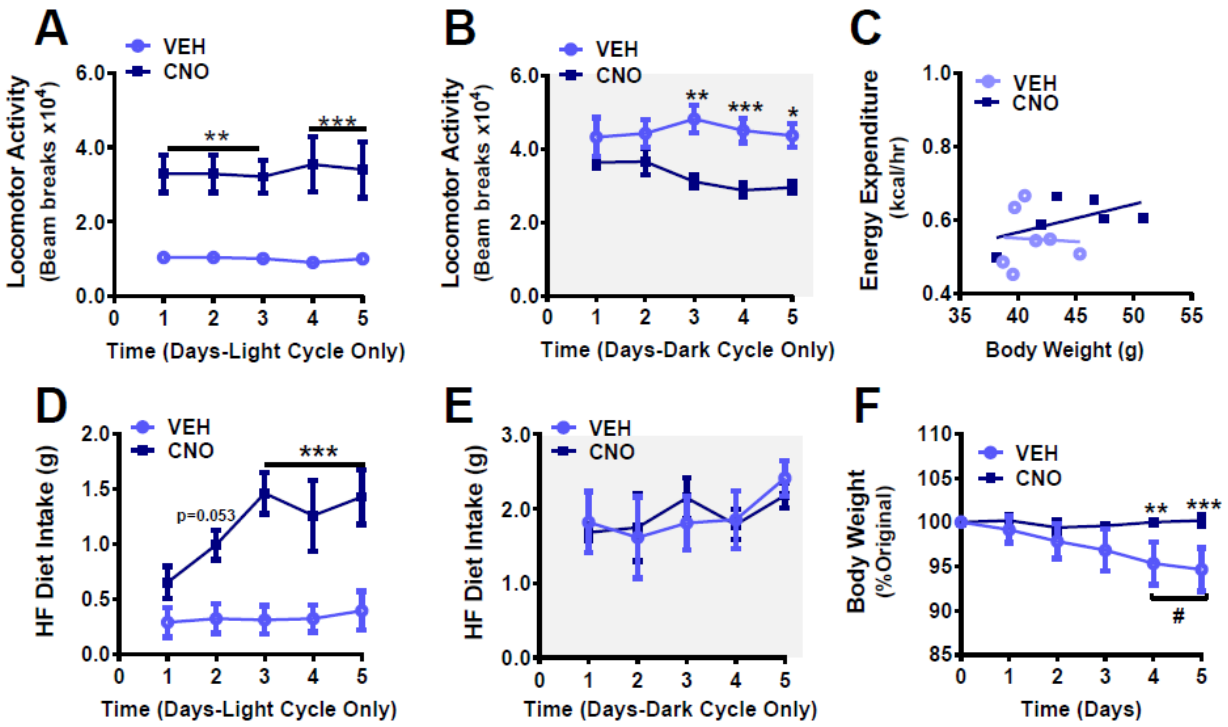

Figure 34. Repeated activation of LHA Nts neurons does not induce weight loss in obesity. LHA Nts neurons were activated each morning for five consecutive days in diet-induced obese mice analyzed in TSE metabolic chambers. **A)** Daily locomotor activity during the light cycle vs. **B)** dark cycle. **C)** Plot showing average rate of energy expenditure vs. body weight. **D)** Food intake during light cycle compared to **E)** dark cycle. **F)** Percent original body weight over five days of chronic VEH or CNO injections. VEH n=6, CNO n=5-6. Data were analyzed by standard two-way ANOVA except for **C** which was analyzed by ANCOVA. (*) indicates significant difference between VEH and CNO at given time point. In **F**, (#) represents significant difference in body weight at days 4-5 compared to day 0 in the VEH group.

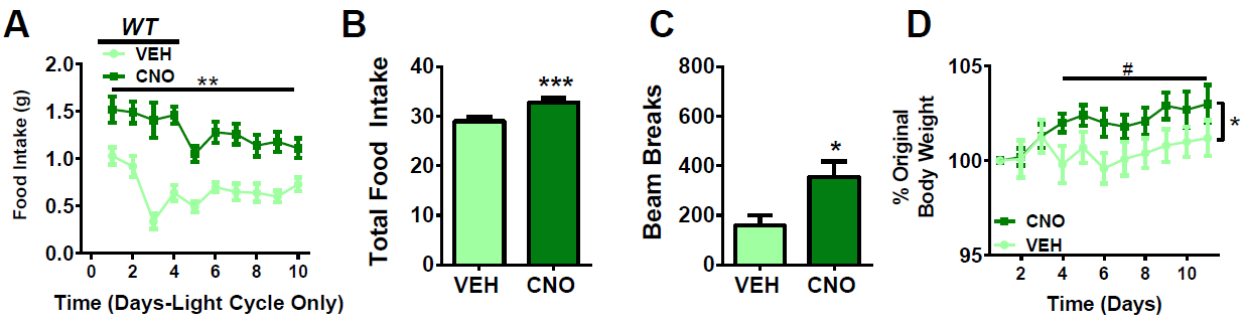

Figure 35. Chronic activation of LHA Nts neurons does not prevent weight gain in lean mice on HF diet. Lean *WT* and *NtsR1KO* mice were injected each morning on VEH or CNO for 10 consecutive days with *ad lib* access to HF diet in home cages. **A)** HF diet intake during the light cycle, **B)** total food consumed over 10 days, **C)** 1 hour locomotor activity, and **D)** body weight in *WT* mice (n=10). (*) represents $p<0.05$ between VEH and CNO from Days 4-10. (#) indicates $p<0.05$ for CNO time point compared to Day 1. Daily food intake and body weight were analyzed by repeated-measures two-way ANOVA. Total food intake and locomotor activity was analyzed by paired t-test.

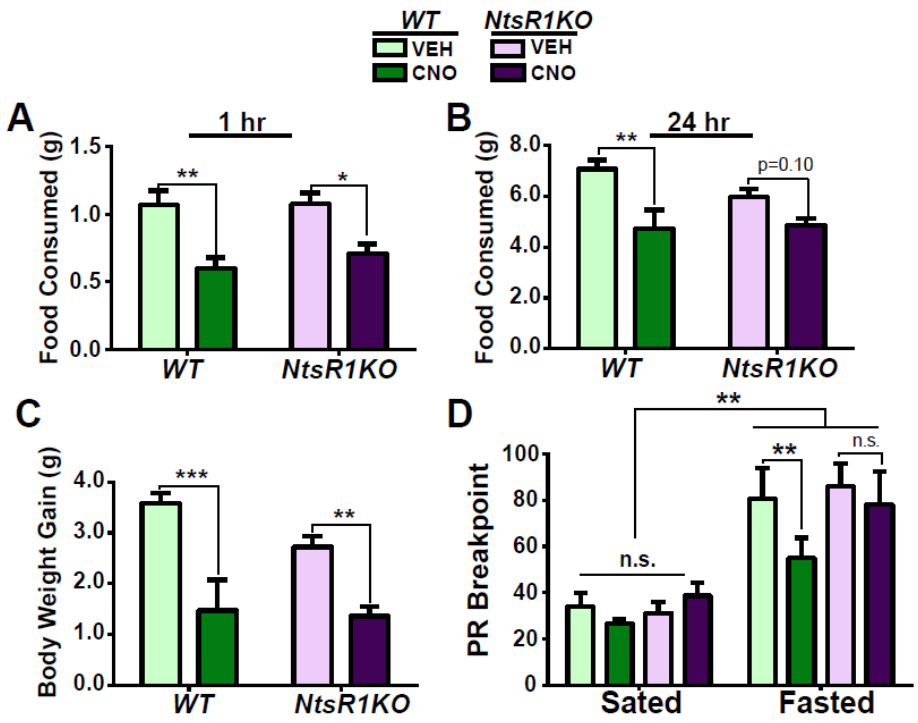

Figure 36. Activation of LHA Nts neurons suppresses fasting-induced *ad lib* and motivated food intake. *WT* and *NtsR1KO* mice were food-deprived overnight and received VEH or CNO with food restoration the following morning. **A, B)** *Ad lib* chow re-feeding 1 and 24 hours after food restoration. **C)** Body weight gain during 24 hours of *ad lib* re-feeding after overnight food-deprivation. **D)** PR breakpoint for sucrose pellets after VEH or CNO injection in both fed and fasted states. *WT* n=9, *NtsR1KO* n=12, data were analyzed by repeated measures two-way ANOVA.

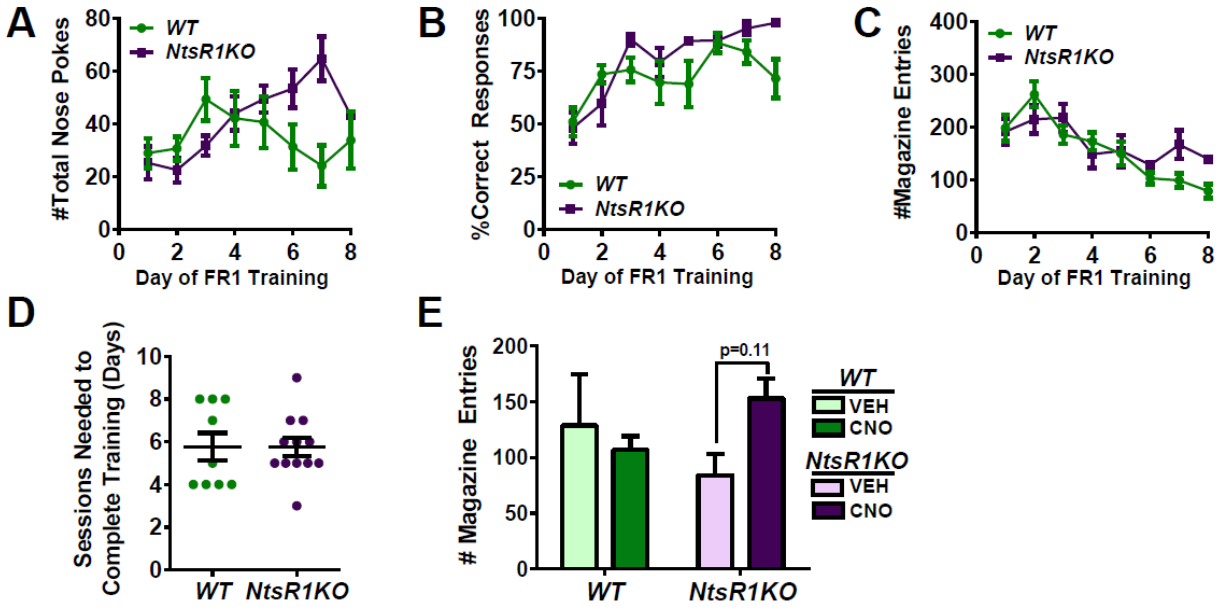

Figure 37. Learning acquisition during operant training. *WT* and *NtsR1KO* mice were trained to nose-poke for sucrose pellets on an FR1 schedule until they earned >20 rewards with 75% accuracy for 3 consecutive days. **A, B, C)** No differences were detected in total number of active and inactive nose pokes, accuracy, and number of magazine during FR1 training. **D)** *WT* and *NtsR1KO* mice required a similar number of training sessions to learn the task. **E)** CNO treatment did not significantly increase the number of magazine entries during PR testing. *WT* n=9; *NtsR1KO* n=12. Data were analyzed by repeated-measures two-way ANOVA **(A,B,C,E)** or unpaired t-test **(D)**.

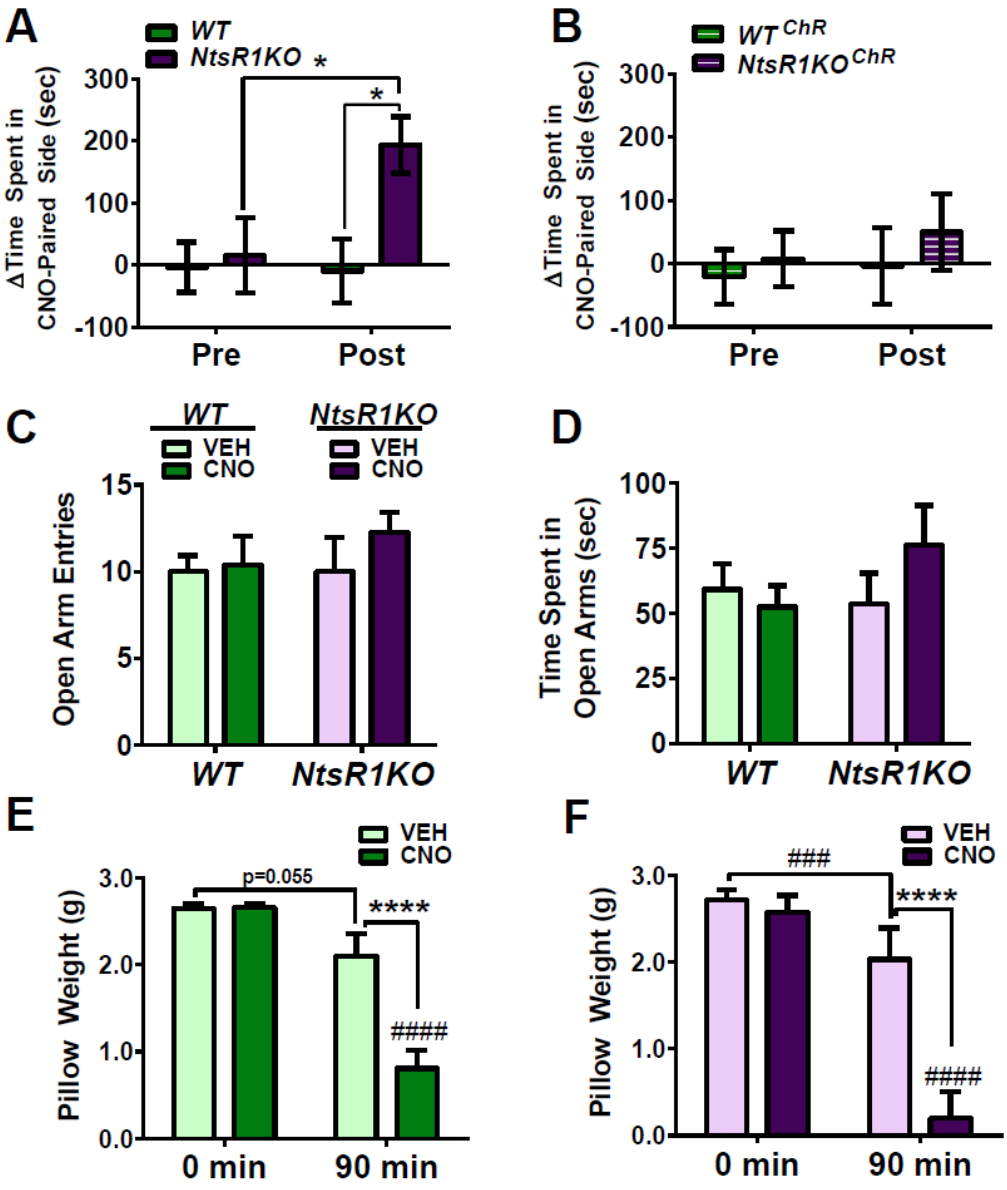

Figure 38. Assessment of reward, anxiety, and repetitive behaviors associated with LHA Nts→NtsR1 circuit activation. CPP was used assess whether activation of LHA Nts neurons could be either positively or negatively reinforcing. **A)** Time-spent in the CNO and VEH-paired sides during the pre- and post-tests reveals that activation of LHA Nts neurons is rewarding in *NtsR1KO* (n=15) but not *WT* (n=17) mice. **B)** No differences were detected in WT^{ChR} (n=12) and $NtsR1KO^{ChR}$ (n=10) controls. Data were analyzed by two-way ANOVA. Anxiety-like behavior was assessed via EPM and no differences were detected in open arm **C)** entries or **D)** time spent (*WT*+VEH n=10, *WT*+CNO n=10, *NtsR1KO*+VEH n=6, *NtsR1KO*+CNO n=8). **E, F)** Nestlet weight 90 minutes after VEH or CNO injection in *WT* and *NtsR1KO* mice (*WT*+VEH n=8, *WT*+CNO n=10, *NtsR1KO*+VEH n=6, *NtsR1KO*+CNO n=8). Nestlet-shredding and EPM data were analyzed by standard two-way ANOVA while CPP was analyzed by repeated-measures two-way ANOVA to compare pre-test to post-test.

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Chapter 6. Summary, Discussion, and Translational Implications

6.1 Summary of Dissertation

The goal of this thesis was to understand how Nts engages mesolimbic DA signaling to modify body weight. In Chapter 2, we established the sources of endogenous Nts input to the VTA, and found that it originates from neurons of the LHA, POA, and NA. This anatomical framework was necessary to reveal which brain regions can coordinate Nts release to the VTA, and hence may utilize Nts signaling to modify energy balance. Next, in Chapter 3, we defined which VTA cells express NtsRs and thus are capable of intercepting Nts to modify DA signaling. We determined that NtsR1 is the predominant Nts receptor isoform on VTA DA neurons, while NtsR2 is instead expressed on glial cells. Furthermore, the subset of VTA DA neurons that express NtsR1 project heavily to the NA and not the PFC. Collectively, these data reveal the cellular mechanism for Nts to engage mesolimbic DA signaling: via binding to NtsR1-expressing VTA DA neurons that exclusively project to, and can release DA into, the NA. We then tested whether these VTA NtsR1-expressing DA neurons are essential for energy balance by genetically ablating them in Chapter 4. Indeed, loss of VTA NtsR1-DA neurons led to chronic hyperactivity and elevated energy expenditure that impaired age- or diet-related weight gain compared to intact controls. Additionally, loss of VTA NtsR1-DA neurons disrupted coordination of motivated feeding behavior with peripheral energy cues, indicating that this VTA DA neuronal subset may serve to adjust feeding behavior in response to peripheral energy status. Finally in Chapter 5, we put these data together to investigate whether LHA Nts neurons that project to the VTA are endogenous mediators of feeding and locomotor behaviors via Nts signaling to NtsR1. Using DREADD technology, we found that activation of LHA Nts neurons suppresses chow feeding in both fed and fasted states via NtsR1 and DA-dependent mechanisms. LHA Nts neurons also promoted increased physical activity and energy expenditure independently of NtsR1. The dual anorectic and activity-enhancing effects of LHA Nts neurons supported both

short and long-term weight loss, but palatability/caloric value can subvert the anorectic effects of the LHA Nts circuit. Importantly, LHA Nts action suppressed motivated sucrose self-administration in hungry animals via an NtsR1-dependent mechanism, which coincides with the ability of Nts to modulate mesolimbic DA signaling via NtsR1, and the specific requirement of mesolimbic DA signaling for reward "wanting", not *ad libitum* feeding (discussed in Chapter 1). Collectively, this work defines a neuronal circuit whereby Nts input from the LHA modifies mesolimbic DA via NtsR1 and restrains feeding in the face of negative energy balance, which may help initiate and maintain weight loss (summarized in Fig. 39).

6.2 Discussion

6.2.1 Technical Considerations of Transgenic vs. Knock-in Models to Study VTA NtsR1 Neurons

When these studies were initiated, the only Cre-driver line available to study NtsR1 was a commercially-available *NtsR1*^{Cre} transgenic model. We therefore began using transgenic *NtsR1*^{Cre} mice to determine the functional requirement for VTA NtsR1 neurons in Chapter 4, while simultaneously working to generate a knock in *NtsR1*$^{Neo\text{-}Cre}$ model. Our rationale for developing a knock-in model was based on their proven utility for reporting endogenous gene expression, particularly for lowly-expressed transcripts such as receptors[1,2]. Transgenic models, however, come with inherent limitations; due to the transgene (i.e. Cre) being inserted randomly into the genome, expression levels tend to be low and may vary across generations[3]. Indeed, when bred to a Cre-inducible GFP reporter, the transgenic *NtsR1*^{Cre} model reported 5% of VTA DA neurons co-expressing NtsR1 (Fig. 18) and the overall number of GFP-labeled neurons was much less than expected compared to *Ntsr1* ISH data from the Allen Brain atlas[4]. By contrast, subsequent examination of the *NtsR1*$^{Neo\text{-}Cre}$ knock-in mice revealed that 70% of VTA DA neurons co-expressed NtsR1 (Fig. 14), more consistent with the Allen Brain distribution of

Ntsr1. Naturally, labeling 5% vs. 70% of cells is an appreciable difference, and suggests that *NtsR1*$^{Neo\text{-}Cre}$ knock-in mice are more advantageous for detecting NtsR1 neurons via breeding to Cre-inducible mouse lines. However, we observed labeling of far more than 5% of DA neurons in the transgenic *NtsR1*^{Cre} model after injection of Cre-inducible viral reporters (Fig. 26). We thus suspect that Cre expression is quite low in most of the NtsR1-expressing cells of the transgenic model and as a result, only a few NtsR1 neurons have sufficient amounts of Cre protein to induce recombination of a Cre-inducible GFP allele necessary to be labeled (5% of TH+ neurons, Fig. 18). By contrast, injection of viral vectors leads to a much higher amount of Cre-inducible reporter in cells, requiring a lower threshold of Cre expression to induce recombination. Thus, transgenic *NtsR1*^{Cre} mice with very low levels of Cre expression are more likely to exhibit Cre-mediated recombination and be labeled after treatment with viral vectors (Fig. 26). Despite the discrepancy between the transgenic and knock-in models when bred to reporter lines, we suspect that injection of the AAV-DTA targeted a substantial number of the true NtsR1+ population (i.e. closer to 70% rather than 5% of TH+ neurons). This is further supported by the anterograde tracing data, which was largely consistent between the transgenic and knock-in models (Fig. 15 vs. Fig. 19), with the knock-in model revealing some additional minor projections to regions outside the ventral striatum that may not have been appreciable in the transgenic line. Going forward, it will be critical to validate the behavioral data in Chapter 4 using Cre-mediated ablation or inhibitory chemogenetics in *NtsR1*$^{Neo\text{-}Cre}$ knock-in mice, which reliably detect the full extent of NtsR1 neurons.

6.2.2 Phenotype of Mice Lacking VTA NtsR1 Neurons

A prominent feature of mice with genetically ablated NtsR1 neurons (Chapter 4) was their persistently elevated volitional locomotor activity. This behavior, and the increased energy expenditure to support it, is likely what protected these mice from weight gain, as their food intake was either unchanged or slightly increased compared to controls with normal activity (Fig.

20). A limitation of ablating neuronal populations is that the entire neuron, including all its neurotransmitters and receptors are abolished, thus resultant behaviors cannot be reliably attributed to one particular signal originating from the neuron. Therefore, we cannot conclude that the hyperactivity or uncoupling of energy balance is due to loss of NtsR1, and Nts action, *per se*. We speculate that the hyperactivity is due to compensatory remodeling of mesolimbic DA circuits that occurs independently of NtsR1. Loss of substantial mesolimbic DA terminals may have potentiated increased DA release and turnover from the remaining intact terminals, causing the DA system to constantly function on overdrive, similar to the action of psychostimulants. In fact, multiple colleagues remarked that the ablated mice look similar to normal mice treated with cocaine, which is characterized by inhibition of DA reuptake and persistently high levels of DA in the synapse. We suspect that if ablated mice were not hyperactive, they may instead have overconsumed palatable food and become overweight due to their impaired ability to respond to satiety cues, an effect that likely *is* dependent on Nts-NtsR1 signaling. In support of this hypothesis, mice constitutively lacking NtsR1 have intact VTA DA neurons, but exhibit an impaired anorectic response to leptin and are prone to weight gain on HF diet, indicating that NtsR1 is required for adjusting feeding in response to satiety cues[5,6]. Loss of neurons expressing NtsR1 selectively in the VTA produced a similar disruption in coordinated feeding behavior, however the increased energy expenditure may have masked any susceptibility to weight gain. In the future, deletion of NtsR1 itself from the VTA, not the entire neuron, will be essential to verifying the necessity of VTA Nts-NtsR1 signaling in coordination of energy balance. Developing floxed NtsR1 mice, and injecting them in the VTA with Cre-inducible reagents to delete NtsR1 from adult VTA NtsR1 neurons could accomplish this, and holds promise to reveal the physiological role of NtsR1 in the adult brain.

6.2.3 Chemogenetic Activation of LHA Nts Neurons: Limitations and Future Directions

In Chapter 5, we demonstrated that activation of LHA Nts neurons suppressed feeding via an NtsR1 and DA-dependent mechanism, suggesting this occurred via an LHA Nts→VTA NtsR1 circuit. While the VTA is the most likely site of Nts-NtsR1 action, the study was designed such that *all* LHA Nts neurons were activated by the chemogenetic strategy, including LHA Nts neurons that may not project to the VTA. Future work will be important to provide more circuit-specific resolution to determine whether or not the anorectic effects of LHA Nts neurons are mediated at the level of the VTA or elsewhere. In addition to suppressing food intake via NtsR1, LHA Nts neurons also increased locomotor activity and energy expenditure by an NtsR1-independent mechanism. Thus, these effects could be mediated by other neurotransmitters released by LHA Nts neurons such as GABA, glutamate, or other neuropeptides, to the VTA or elsewhere. To determine whether behavioral effects are specifically mediated by the VTA, CNO could be infused directly into the VTA of *WT* and *NtsR1KO* mice. Injecting drugs through cannulae would be more technically challenging than *i.p.* injections, especially for studies requiring mice to be treated multiple times over the course of an experiment. Additionally, generating study animals would be more difficult, as this would require both bilateral targeting of AAV to the LHA *and* of cannulae to the VTA. An alternative approach would be to design a two-step retrograde Cre-inducible DREADD virus that traveled monosynaptically, whereby injection into the VTA would allow the virus to travel to Nts neurons that project to the VTA and a second injection of a helper virus into the LHA would permit DREADD receptor expression only in LHA Nts neurons that project to the LHA, similar to modified rabies viruses[7].

While we experimentally activated *all* LHA Nts neurons, they may not all contribute to the regulation of energy balance. Indeed, LHA Nts neurons can be divided into functionally distinct subpopulations. For example, ~15-30% of LHA Nts neurons are regulated by leptin, while a separate group is selectively regulated by dehydration[2,8,9]. Thus general activation of *all* LHA

Nts neurons may produce a mixed phenotype due to the simultaneous actions of feeding-specific and drinking-specific subsets. We suspect that the anorectic effects of LHA Nts neurons are likely mediated by the subset of LHA Nts neurons that express LepRb, while the drinking behavior is induced by the dehydration-sensitive LHA Nts neurons. Since all of these neurons are activated by peripheral CNO, some of the behavioral findings may be complicated by desire to drink, since animals did not have access to water in the operant chambers or CPP boxes. Per the sucrose self-administration studies, this is likely not the case as activation of all LHA Nts neurons did not alter baseline PR breakpoint and was only effective at reducing responding in a fasted state (Fig. 36D). Animals had free access to water during the overnight fast and although they may have consumed less, it is unlikely that slight dehydration and/or desire to drink suppressed motivation for sugar pellets; it is much more likely that increased activation of the leptin-sensitive LHA Nts subset is responsible for reduced operant responding in a fasted state, similar to a peripheral injection of leptin[10].

From a translational perspective, an important finding was that activation of LHA Nts neurons was neither rewarding nor aversive in the presence of NtsR1 (Fig. 38A). Hence, our studies suggest that an NtsR1 agonist, preferentially delivered to the VTA, could restrain appetite in a food-deprived state to help initiate and maintain weight loss but without promoting dependence. It was vital to address the potential addictive nature of activating Nts-NtsR1 signaling, given that it increases mesolimbic DA release[11,12] similar to addictive drugs like cocaine and amphetamine. Indeed, prior work suggested that pharmacologic administration of Nts to the VTA is positively reinforcing[13-15], but this could have been due to high, non-physiologic concentrations of Nts tested. A novel anti-obesity agent with abuse potential would not be clinically viable. However, our data suggest that activating LHA Nts neurons, and hence endogenous Nts release, may act via a specific subset of VTA DA neurons that either do not

functionally promote positive reinforcement or simply does not cause sufficient DA release to be reinforcing.

We found that activation of LHA Nts neurons suppresses feeding in hungry, fasted mice. To clarify the translational potential of this circuit, a series of critical follow-up experiments will be necessary. A key future experiment would be to calorically restrict diet-induced obese mice (i.e. put them on a "diet") and after initial weight loss, activate LHA Nts neurons in the presence of *ad libitum* chow or HF diet to see if increased action of the circuit can prevent weight regain in weight-reduced obese mice. This experiment mirrors the metabolic circumstances faced by obese individuals who struggle to maintain weight loss in the face of negative energy balance. If this experiment shows that LHA Nts neurons support sustained weight loss over time, then the circuit holds strong translational potential for maintaining reduced bodyweight in previously overweight individuals. It is possible however, that the LHA Nts circuitry is altered after the onset obesity, as suggested by the inability of LHA Nts neuronal activation to induce weight loss in well-fed obese mice (Chapter 5, Fig. 34). If this is the case, then the LHA Nts circuit may still hold promise in in preventing weight gain or maintain weight loss in younger individuals who have not had long-standing obesity but are at risk for continued weight gain.

6.3 Translational Implications

6.3.1 Neurotensin Circuits as a Translational Target

Although this work provides preliminary evidence that LHA Nts→NtsR1 signaling may be a candidate anti-obesity target, several questions and challenges need to be addressed to fully understand the function of this circuit in weight control. First, the circuit needs to be tested in the presence of additional variables representative of human populations, including gender, age, and genetic background. All studies in Chapter 5 were performed with male mice with

mixed genetic backgrounds (C57/Bl6 and 129 strains) and although testing occurred at a variety of ages, the mice were always injected with DREADD virus between 8-12 wk of age, thus the expression of DREADD receptors would have been similar across the lifespan. It will also be important to know whether LHA Nts or VTA NtsR1 expression varies with age or gender. We did not observe any overt differences in NtsR1 expression across gender or age (Chapter 3), but these studies were not designed to interrogate those variables. Importantly, we must understand whether the Nts→NtsR1 system is anatomically present in humans, a question that is difficult to answer given the inability of immunohistochemistry to reliably detect Nts and NtsR1-expressing cell bodies. To date, one study examined Nts immunoreactivity in the human brain and reported Nts expression in both the LHA and VTA[16], however more work with human tissue is necessary to confirm similarity in the LHA Nts→ VTA NtsR1 circuit across species.

Nts has been investigated as a drug target for both schizophrenia and Parkinson's disease. In 2004, the results of a clinical trial testing the efficacy of an NtsR1 antagonist (SR48692 or meclinertant) for schizophrenia found no differences in symptom improvement compared to placebo[17]. In a letter to the editor, Richelson *et al.* expressed that they were not surprised by the lack of efficacy of an NtsR1 antagonist and proposed instead that a brain penetrating non-peptide NtsR1 agonist which they had developed and intended to use in clinical trials would be more effective[18]. However, the fate of this non-peptide agonist is unclear. An Nts analogue (NT69L) and an NtsR1 agonist (PD149163) have been used routinely in animals, however data regarding testing of these agents in clinical trials is either unavailable or non-existent. Interestingly, a conjugated Nts nanoparticle has been used to deliver neuroprotective genes specifically to DA neurons in animal models of Parkinson's disease[19,20]. This approach takes advantage of the internalization of Nts-NtsR1 receptor complexes, and thus genes packaged and tagged to a modified Nts peptide are specially targeted to DA neurons, which have the high levels of NtsR1 expression. While this method proves promising in preclinical

studies, the nanoparticle has only been administered directly to the SN, and it is unclear whether or not the particle would have the same effect if administered orally or peripherally. While this approach could be used to enhance Nts-NtsR1 signaling specifically on DA neurons, which could have potential as an anti-obesity agent based on this dissertation, a considerable challenge would be developing a drug with minimal peripheral side effects. As discussed in Chapter 1, peripheral Nts promotes hypotension and increased absorption of dietary fat, which could provide undesirable side effects[21,22]. Interestingly however, Nts-NtsR1 signaling is a candidate drug target for a wide variety of cancers due to its role in tumor growth, and currently, meclinertant (the NtsR1 antagonist) is being assessed in a clinical trial for lung cancer (ClinicalTrials.gov identifier NCT00290953). Thus, although an NtsR1 agonist would be desirable for weight-loss, perhaps Nts-targeted agents developed through these pipelines may also prove useful in controlling body weight, and underscores the utility of idea-sharing across disciplines among medical professionals and scientists.

6.3.2 Final Thoughts on the Challenges of Addressing Obesity

From the perspective of a future scientist and healthcare provider, the challenges posed by the obesity epidemic are humbling. The standard first-line treatment recommended by physicians is diet and exercise, despite overwhelming evidence that diets do not succeed in the long term[23]. Thus when patients fail to maintain weight loss, many feel tremendous guilt due to perceived lack of will-power to avoid high calorie foods. Patients are often *not* told that strong biological mechanisms, not personal weakness, are driving them to overconsume food and regain the weight. Biology drives behavior, and unfortunately many people falsely assume that obese individuals are too lazy or unmotivated to lose weight, attributing their excess weight to personal flaw. This is simply not the case. Perhaps better education of both patients and healthcare providers in the science of energy balance and weight loss could help alleviate the negative stereotypes and emotional guilt experienced by obese individuals. While it seems

clear that diets are not the answer, this leaves a giant gap in the treatment options for obesity. This is where scientists have enormous opportunity to develop better treatment options, which I hope will be fueled by the advent of optogenetic and pharmacogenetic techniques to decipher neural feeding circuits. Ultimately, addressing the multi-factorial nature of obesity will require concerted effort from medical professionals, scientists, policy-makers, and public health advocates. Although the stakes are high, the future holds promise for a solution to the obesity crisis.

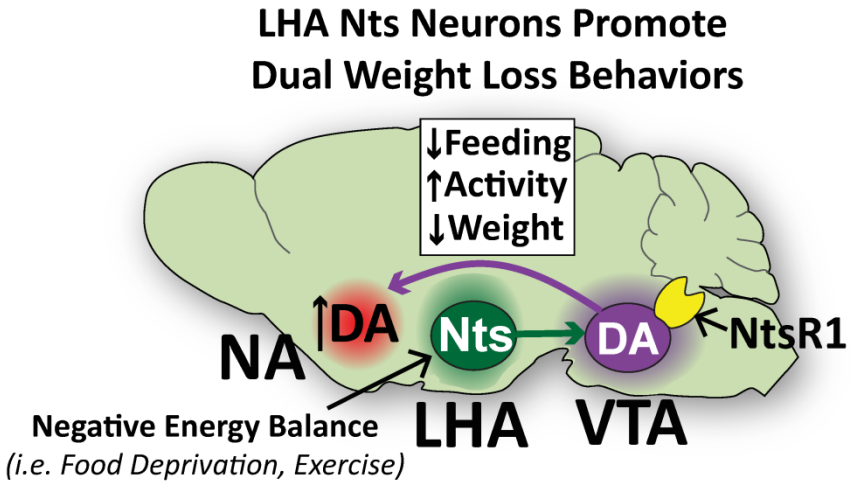

Figure 39. LHA Nts neurons restrain feeding and promote locomotor activity, in part via NtsR1 and the mesolimbic DA system. Enhanced activation by the LHA Nts circuit may be useful to support weight-loss in weight-reduced individuals.

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