ROLE OF VENTRAL TEGMENTAL AREA NEUROTENSIN RECEPTOR-1 NEURONS IN ENERGY BALANCE

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ABSTRACT

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Dopamine (DA) neurons in the ventral tegmental area (VTA) modulate physical activity and feeding behaviors that are disrupted in obesity. Although the heterogeneity of VTA DA neurons has hindered determination of which ones might be leveraged to support weight loss, we have characterized a subset of VTA DA neurons that express NtsR1 (VTA NtsR1 neurons) that are involved in the coordination of energy balance. We hypothesized that 1) increased activity VTA NtsR1 neurons might promote weight loss behaviors, and that 2) deleting NtsR1 specifically from VTA DA neurons would promote weight gain by increasing food intake and decreasing physical activity. We first used Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to activate VTA NtsR1 neurons in normal weight and diet-induced obese mice. Acute activation of VTA NtsR1 neurons (24hr) significantly decreased body weight in normal weight and obese mice by reducing food intake and increasing physical activity. Moreover, daily activation of VTA NtsR1 neurons in obese mice sustained weight loss over 7 days. Activating VTA NtsR1 neurons also suppressed how much mice worked to obtain sucrose rewards, even when there was high motivation to consume. However, VTA NtsR1 neural activation was not reinforcing, nor did it invoke anxiety, vasodepressor responses or hypothermia. We then used newly generated *NtsR1*$^{flox/flox}$ mice to study NtsR1 deletion in both development and adulthood. Curiously, developmental deletion of VTA NtsR1 (by crossing *DAT*^{Cre} mice with *NtsR1*$^{flox/flox}$ mice) had no impact on feeding or body weight. Adult deletion of the receptor (by injecting adeno

associated Cre into VTA of adult *NtsR1*$^{flox/flox}$ mice), however, resulted in lower body weight and DA-dependent food intake. Altogether, these data suggest that modulating NtsR1 expression in the adult VTA may be useful to safely promote weight loss, and that NtsR1 is worth further exploration for managing obesity.

To my younger self;
without her courage and perseverance, I would not have made it this far.

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TABLE OF CONTENTS

LIST OF FIGURES

KEY TO ABBREVIATIONS

AMPH	:	Amphetamine
ANOVA	:	Analysis of Variance
BMI	:	Body Mass Index
CAR	:	Campus Animal Resources
CNO	:	Clozapine-N-Oxide
CNS	:	Central Nervous System
CPP	:	Conditioned Place Preference
CRF	:	Corticotropin Releasing Factor
CRFR1	:	Corticotropin Releasing Factor Receptor-1
CRFR2	:	Corticotropin Releasing Factor Receptor-2
DA	:	Dopamine
DAT	:	Dopamine Transporter
DREADDs	:	Designer Receptors Exclusively Activated by Designer Drugs
EPM	:	Elevated Plus Maze
FR	:	Fixed Ratio
Gal	:	Galanin
GPCRs	:	G-Protein Coupled Receptors
HFD	:	High Fat Diet
LepRb	:	Leptin Receptor - long form
LHA	:	Lateral Hypothalamic Area

MCH	:	Melanin Concentrating Hormone
NAc	:	Nucleus Accumbens
Nts	:	Neurotensin
VTA	:	Ventral Tegmental Area
VEH	:	Vehicle/Physiological Saline Solution
NtsR1	:	Neurotensin Receptor-1
NtsR2	:	Neurotensin Receptor-2
OX/HCRT	:	Orexin/Hypocretin
OXR-1	:	Orexin Receptor-1
OXR-2	:	Orexin Receptor-2
SNc	:	Substantia Nigra Pars Compacta
PR	:	Progressive Ratio
TH	:	Tyrosine Hydroxylase

CHAPTER 1. LATERAL HYPOTHALAMIC AREA NEUROPEPTIDES MODULATE VENTRAL TEGMENTAL AREA DOPAMINE NEURONS AND FEEDING

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ABSTRACT

Understanding how the brain coordinates energy status with the motivation to eat is crucial to identify strategies to improve disordered body weight. The ventral tegmental area (VTA), known as the core of the mesolimbic system, is of particular interest in this regard because it controls the motivation to consume palatable, calorie-dense foods and to engage in volitional activity. The VTA is largely composed of dopamine (DA) neurons but modulating these DA neurons has been alternately linked with promoting and suppressing feeding, suggesting heterogeneity in their function. Subsets of VTA DA neurons have recently been described based on their anatomical distribution, electrophysiological features, connectivity, and molecular expression, but to date there are no signatures to categorize how DA neurons control feeding. Assessing the neuropeptide receptors expressed by VTA DA neurons may be useful in this regard, as many neuropeptides mediate anorexic or orexigenic responses. In particular, the lateral hypothalamic area (LHA) releases a wide variety of feeding-modulating neuropeptides to the VTA. Since VTA neurons intercept LHA neuropeptides known to either evoke or suppress feeding, expression of the cognate neuropeptide receptors within the VTA may point to VTA DA neuronal mechanisms to promote or suppress feeding, respectively. Here we review the role of

the VTA in energy balance and the LHA neuropeptide signaling systems that act in the VTA, whose receptors might be used to classify how VTA DA neurons contribute to energy balance.

1.1 THE IMPORTANCE OF FEEDING AND ENERGY BALANCE

Perhaps the most frequent behaviors that animals engage in are eating and drinking. The physiological processes required for life (e.g., thermogenesis, respiration, waste filtration, movement etc.) constantly consume onboard energy and water, so these resources must be replenished. Since energy from food and water can only be obtained via ingestion, survival depends on detecting the need for these resources and motivating the behavior to seek and consume them. Multiple neural circuits that contribute to feeding have been characterized, and serve to tune feeding to the environmental, interoceptive and emotional state of the organism[1,2]. Yet, despite the fundamental necessity of ingestion, the neural mechanisms by which the brain modulates the motivation to seek and consume food are yet to be fully elucidated. Understanding these processes is paramount for understanding basic biology and survival.

Feeding implicitly impacts "energy balance", a term used to describe the linked relationship of energy intake and energy expenditure that has important ramifications for health. Energy intake refers to the calories obtained from ingested food and fluids, while energy expenditure refers to the energy that is consumed through basal metabolic rate, thermogenesis and physical activity[3]. Body weight is a "read-out" of energy balance, since it

depends on the number of calories consumed to calories burned. Changes in the extent of feeding or energy expended accordingly change body weight. There are periods when it is advantageous to be "imbalanced". For example, during development, more calories must be consumed than expended to support growth, including increased body weight. In adulthood, energy balance should be more stable, such that day to day energy intake and output are relatively equal, and weight is maintained[3]. Even in this state, however, dynamic fluctuations of energy intake occur across the day as energy consumption creates need to replenish energy reserves. Energy deficiency registers as hunger and increases the drive to find and ingest food. Feeding restores satiety, which reduces the drive to eat while enabling for locomotor activity, thermogenesis and other energy dependent behaviors[3]. These actions are largely orchestrated by neurons in the hindbrain and mediobasal hypothalamus, collectively referred to as the homeostatic system. Thus, cues of energy status inform the body about food "need" and promote appropriate behaviors to resolve any energy imbalance.

Yet, feeding may also occur in the absence of need, and can threaten energy balance. For example, despite being satiated from a meal we might still eat dessert simply because it tastes good. Hence, external cues and/or the anticipated reward from food can potentiate excess caloric intake, and accordingly, weight gain. Overweight and obesity are energy balance disorders that develop due to elevated consumption of palatable and calorie-dense foods. Since overconsumption often occurs in combination with sedentary lifestyle and reduced energy expenditure, this leads to a "perfect storm" for weight gain[4]. More than one-third of US adults are obese, placing them at increased risk to develop chronic co-morbidities such as

cardiovascular diseases and type-2 diabetes that diminish life quality and length[5,6]. Curbing so called “reward feeding” is therefore a major goal to treat and prevent the development of overweight and obesity. While there are some pharmacotherapies to support acute weight loss, they are minimally effective in the long-term to restrain feeding, and have yet to stem the rising disease incidence[4,7,8]. As a result, combined diet and exercise remain the most widely prescribed treatment for overweight and obesity. However, dieting increases appetitive drive that can spur overeating such that most individuals regain weight with time[9–13]. Since altered motivation and drive to eat negatively impacts health it is vital to understand how the brain coordinates feeding behavior, to pinpoint how and why it goes awry in disease states.

1.2 THE VENTRAL TEGMENTAL AREA MODULATES FEEDING

The ventral tegmental area (VTA) is well known to modify intake of pharmacological and natural rewards, including food. While the neural mechanisms of this process remain incompletely understood, the VTA appears to coordinate energy status and external cues so as to influence feeding behavior. The VTA is located near the base of the midbrain and it contains dopamine (DA) neurons (~60%-65%), GABA neurons (~30%-35%) and a small subset of glutamate neurons (~2%-3%)[14,15]. Given these proportions, it is of little surprise that most research has focused on the role of the DA neurons within the VTA and their roles in feeding behaviors. However, recent work confirms that some subpopulations of VTA GABA neurons[16–18] and VTA glutamate neurons[19–21] can modify behaviors independently of DA neurons, some of which are relevant to feeding. VTA glutamate neurons are located primarily in the rostral and

medial portions of the VTA, and their activation has been shown to drive conditioned place preference, reinforcement of instrumental behavior and aversive conditioning in mice[19–21]. GABA expressing neurons also have a role in aversion[17,22], but, on the other hand, are distributed throughout the VTA[16,23,24] and have been shown to suppress the activity and excitability of neighboring DA neurons[16].

Owing to their abundance, however, VTA DA neurons are the most studied VTA population, and are involved in an array of motivated behaviors[22,25–27], from positive and negative reinforcement, decision making, working memory, stimulus salience and aversion[28–33]. VTA DA neurons also respond to cues that predict rewards[34] and DA is a key substrate in the incentive and reinforcing aspects of food intake[35–39], locomotor activity and body weight[40]. This participation of VTA DA neurons in so many actions suggests a degree of functional heterogeneity, and has prompted speculation on whether there are subsets of DA neurons to mediate specific behaviors – including, perhaps, specific feeding behaviors. In potential support of this idea, experimental activation of VTA DA neurons has yielded a "mixed bag" of feeding responses, from enhancement to suppression[41–44]. One possible explanation may be the methods used to target and activate the VTA DA neurons, which at least in some cases only activated a portion of all VTA DA neurons[41,42]. It is possible, therefore, that these experiments manipulated subsets of VTA DA neurons that differ in how they control feeding. Similarly, while not examining feeding specifically, recent work demonstrates that modulating the activity of subsets of VTA DA neurons differentially modifies behavioral reinforcement[44]. Moreover, manipulation of specific subsets does not always produce the same behavior as manipulation of

all VTA DA neurons[44]. In combination, these findings support examination of methods to classify VTA DA neurons, to identify whether there are subsets with different roles in regulating feeding.

There has been a recent explosion of work directed toward identifying subsets of VTA DA neurons. They have been geographically classified by their distribution across the VTA; DA neurons in the ventral VTA for example, are excited by noxious foot shocks, in contrast to dorsal VTA DA neurons, which are inhibited[30,45]. VTA DA neurons can also be differentiated according to their electrophysiological firing properties[46,47]. As of yet, however, there is no consensus on specific electrophysiological or anatomical signatures that predict how VTA DA neurons modify feeding. However, VTA DA neurons can also be described by their anatomical inputs and projections[25,44,48], and the latter may provide insight into functional subsets and feeding control. The majority of VTA DA neurons project to the nucleus accumbens (Nac), where DA release has been shown to control the "wanting" of pharmacological and natural rewards and the goal-directed behaviors to obtain them (for review on VTA DA innervating Nac and its influence in motivation see Salamone and Correa, 2012)[33]. Other VTA DA neurons project to the prefrontal cortex, amygdala, hippocampus and other brain regions that alter top-down control, anxiety and learning, all of which can impact the extent of feeding[49]. Given this range in projection targets, it seems plausible that the differences in VTA DA neuronal connectivity might track with separate functionality, and possibly differentiable molecular signatures. Recent work supports that there is variable gene expression across VTA DA neurons,

and the existence of subpopulations with differing expression of transcription factors, channels, DA related genes and receptors for neurotransmitters, hormones and neuropeptides[50–52].

Of these, peptide receptors have garnered considerable interest to parse VTA DA neurons that might influence feeding. Peptide signals can reach the VTA via two routes: via the circulation (as peptide hormones) and through being released from neurons (as neuropeptides). For example, circulating cues of energy status, such as the peptide hormones insulin, leptin and ghrelin act directly upon hypothalamic neurons as well as on VTA neurons[53,54]. Expression of these hormone receptors on VTA DA neurons can identify subsets of neurons that likely contribute to anorectic or orexigenic responses. Yet, hypothalamic regions have more rapid access to circulating cues than the VTA, due to being adjacent to the "leaky" median eminence that permissively allows circulating factors access to the brain. By contrast, the VTA lies deep within the brain and far from the median eminence. Hence, the VTA is capable of directly responding to circulating peptide cues of energy status, but is not considered to be the "first-responder" to them that can rapidly inform the direction of feeding.

The VTA is also modulated by neurons in other brain areas that release neurotransmitters and neuropeptides to VTA neurons. In order for VTA neurons to intercept these released messages they must express the cognate receptors. For example, VTA neurons express classical neurotransmitter receptors, and receive excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmitter input from various areas in the brain (for a review on specific glutamatergic or GABAergic inputs onto specific neuronal populations in the VTA see

Morales and Margolis, 2017)[46]. GABA and glutamate are important modulators of VTA DA neuronal activity but are also critical in regulating a wide array of physiology and aren't solely linked with changing feeding. By contrast, some neuropeptides are directly implicated in modulating feeding, may be anorectic or orexigenic in nature, and mediate these actions via their cognate peptide receptors. Indeed, reports have begun to emerge that classify subsets of VTA DA neurons via their expression of neuropeptide receptors, and at least some of these track with specific projection targets and control of behavioral reinforcement[44,55]. These early studies have only categorized neuropeptide-receptor expressing neurons without assessing the role of the neuropeptide signals on VTA DA subsets nor from where the neuropeptides originated. Defining the neurons that release feeding-modulating neuropeptides to the VTA, as well as which VTA DA neurons respond to them, holds promise to reveal the mechanisms underlying feeding.

1.3 THE LATERAL HYPOTHALAMIC AREA ENGAGES THE VTA TO MODIFY FEEDING

The VTA receives neuronal projections from various areas in the brain, but the three main input contributors are the ventral pallidum, dorsal raphe nucleus and hypothalamus[56,57]. Of all of the subregions of the hypothalamus, the VTA receives its densest inputs from the lateral hypothalamic area (LHA)[56], a region well-studied for its role in energy balance and neuropeptide expressing neurons[58]. The LHA is viewed as a coordinating center, since it receives afferent input concerning internal status (e.g., energy, osmolality, temperature, pH, etc.) and sends projections to brain sites capable of modifying behavior to address any

homeostatic deviations (for a review of the LHA control of energy balance, see Kurt, Woodworth and Leinninger, 2017). While the LHA projects widely throughout the brain to orchestrate homeostasis, the LHA→VTA connection is specifically linked with coordinating energy sensing with goal-directed feeding behavior. Indeed, lesion of either the LHA or the VTA cause similar deficits in feeding, while stimulation of either site can incite ingestion[59–65]. Moreover, LHA neurons directly and indirectly receive cues of energy status, and then accordingly modulate the activity of target neurons (including those in the VTA) to modify feeding[66]. Thus, the LHA works in concert with the VTA to coordinate energy sensing and ingestion[49,56,57].

The nature of LHA communication with the VTA is complex, however, since the LHA exhibits a high degree of cellular heterogeneity. There are multiple molecularly distinct neuronal subpopulations within the LHA that are implicated in the control of diverse physiology, including sleep/arousal, reward, food intake, liquid intake, locomotor activity, stress, and response to inflammation[67]. LHA neurons can be molecularly classified by their transmitters, and typically contain one classical neurotransmitter (either excitatory glutamate or inhibitory GABA) along with several neuropeptides[68–72]. Both of these types of signals can be released to target neurons in the VTA, and hence LHA classical transmitters and neuropeptides can impact VTA-mediated behaviors[54]. This leads to enormous complexity in assessing how any "LHA neuron" that projects to the VTA actually modulates feeding, as it could simultaneously release a mix of signals that mediate different effects. Intriguingly, at least some projection-specified subsets of VTA DA neurons respond differently to the same LHA-derived signals,

though the mechanism of how this occurs is yet unclear[73]. One possibility is that subsets of VTA DA neurons differentially express receptors for classical transmitters or neuropeptides or express them in different proportions that influences what message is received. Since neurotransmitter and neuropeptide signals differ in important ways, including their mechanism and site of release, it is important to consider these features in regard to how LHA signals modulate VTA neurons.

Classical neurotransmitters are released at the active zone of the axon terminal, then diffuse across the synaptic cleft (a magnitude of nanometers) to bind to ionotropic receptors on the post-synaptic neuron. Classical transmitter binding to these receptors mediates rapid changes in ionic current and activity of the postsynaptic neurons. Signaling is terminated by enzymatic degradation of the classical transmitter or active reuptake, either of which reduce the amount of transmitter in the synaptic cleft available to bind to postsynaptic receptors. LHA classical neurotransmitter input to the VTA can support a variety of behaviors, depending on context. For example, experimental activation of all LHA GABAergic neurons that project to the VTA promotes feeding[72,74]. However, LHA GABA neurons show heterogeneity in their neuronal responses during food seeking and consumption[75], suggesting that not every GABA neuron is functionally the same. Indeed, it is now clear that there are multiple subpopulations of GABA neurons within the LHA that differ in their co-expressed neuropeptide content[76]. Moreover, subsets of LHA GABA neurons appear to be activated by distinct peripheral stimuli[77]. Thus, while many LHA neurons that project to the VTA release GABA, it is physiologically unlikely that all LHA GABA neurons are activated at the same time, or that they all exert the same effects

within the VTA. The LHA also contains glutamatergic neurons, some of which project to the VTA and can stimulate feeding and arousal[67,78–80]. However, many LHA glutamatergic neurons project to the lateral habenula, and their stimulation suppresses food intake[81]. Thus, the effect that an LHA neuron will have on the VTA or feeding behavior cannot be predicted by its classical transmitter content alone. Hence, the expression of GABA or glutamate receptors are not likely to distinguish VTA DA neurons and their control of feeding.

The neuropeptide content of LHA neurons is a more reliable predictor of how they engage the VTA and modulate feeding. Indeed, LHA subpopulations can be differentiated by their neuropeptide expression, which hints that they exert distinct, neuropeptide-determined effects on target neurons expressing the cognate receptors. For this reason, LHA neurons are typically referred to by their neuropeptide content, such as "Orexin/Hypocretin neurons (OX/HCRT)" or "Melanin Concentrating Hormone (MCH)" neurons. Although classical neurotransmitters and neuropeptides can be released by the same neurons, they are fundamentally different signaling molecules. (For a review on neuropeptides and their involvement in modulating the central nervous system, see van del Pol, 2012.) Briefly, neuropeptides are short sequences of amino acids (3-36) that are synthesized in the soma[82,83]. They are packaged into dense core granules for release, which can occur at any part of the membrane and is not restricted to the active zone (as is the case for neurotransmitters)[82]. In addition, neuropeptides can diffuse much further than classical transmitters, in the magnitude of microns. Neuropeptides are also thought to have a long extracellular half-life, and so can have prolonged actions on target neurons[82]. Generally, neuropeptides bind to G protein-

coupled receptors (GPCRs) that can be expressed on soma, dendrites, and axon terminals, depending on receptor subtype. Neuropeptide binding to receptors modulates intracellular signaling pathways and phosphorylation of several target proteins that can lead to long-term changes in gene transcription and neuronal function[82]. They can also impact the likelihood of a neuron to be activated in response to classical transmitters[82]. Thus, neuropeptides are considered neuromodulators because their release can broadly affect many neurons in the vicinity and invoke lasting changes in function and activity. However, there are numerous neuropeptides, and each can differentially regulate distinct populations of neurons and behaviors. For example, LHA neuropeptides such as MCH, OX/HCRT and galanin promote feeding, whilst the neuropeptide neurotensin (Nts) suppresses feeding[58]. This range of neuropeptide-regulation indicates that they utilize different mechanisms to modify feeding. Because VTA neurons receive and can respond to these neuropeptides, it is possible that the distribution of neuropeptide receptors on VTA neurons could be used to distinguish VTA populations that differentially modify feeding behavior.

To be clear, neuropeptides are not just limited to the LHA, as they are expressed in many neuronal populations throughout the brain[82]. Moreover, neuropeptides throughout the brain have been implicated in disorders of excessive consumption, including drug and alcohol abuse and eating disorders[83], and at least some of their effects are mediated via the VTA. However, it is notable that many LHA neuropeptide-expressing neurons have been shown to modulate behavior via directly engaging the VTA, including feeding, drinking and physical activity. The varied neuropeptide release from LHA neurons to the VTA indicates a potential

mechanism by which target VTA DA neurons might differ: in their expression of the cognate neuropeptide receptors. Some subsets of VTA DA neurons have already classified by their expression of a neuropeptide receptor, and some of these are receptors for neuropeptide ligands released from the LHA[44,55]. However, it is unlikely that subpopulations of VTA DA neurons are defined by a single neuropeptide receptor. Indeed, VTA DA neurons almost certainly receive multiple, simultaneous peptidergic inputs from the LHA and/or other areas, via which they coordinate dopaminergic output in target regions and modulation of behaivor[54]. However, it is possible that some VTA DA neurons might express a suite of receptors that are indicative of how they modify feeding behavior. Thus, deciphering the neuropeptide signaling systems that mediate hypothalamic-VTA interactions could provide insight into how the brain coordinates energy need and ingestive behavior. Below, and summarized in Figure 2, we review the evidence for LHA neuropeptide regulation of the VTA, given the known importance of the LHA→VTA connection to feeding behavior. Specifically, we address which of the associated neuropeptide receptors might discriminate VTA DA subsets with unique contributions to feeding and body weight.

1.4 LATERAL HYPOTHALAMIC AREA NEUROPEPTIDES THAT MODULATE VTA DA NEURONS

1.4.1 ***Orexin/Hypocretin***

LHA neurons expressing the neuropeptides orexin/hypocretin (OX/HCRT) project widely throughout the brain, including to VTA DA neurons[84]. Interestingly, OX/HCRT is not a stimulator

of food intake *per se*, but a stimulator of arousal, which in turn promotes feeding[66,85–87]. OX/HCRT promotes intake of natural and drug rewards via actions on VTA neurons[69,88–92]. Indeed, OX/HCRT neurons are activated during cue-induced feeding, and they in turn activate VTA DA neurons and promote DA release into the Nac and PFC[69,88,89]. OX/HCRT signaling in the VTA promotes neuronal activity by enhancing excitation and suppressing inhibition of DA neurons[69,93,94]. This promotes DA release necessary to drive reward seeking[89–92]. Thus, OX/HCRT acts via modulating VTA DA neurons to promote the ingestion of highly salient substances, including goal-directed responding for palatable foods. These responses are attenuated by pharmacologically antagonizing OX/HCRT receptors, indicating that the OX neuropeptide is a key signal form OX/HCRT neurons that is necessary to promote feeding (**Figure 2**). There are two identified receptors for OX/HCRT, the G-protein coupled receptors, OX Receptor-1 (OXR-1) and OX receptor-2 (OXR-2). These receptors are expressed throughout the brain, including the VTA, where they are typically coupled to Gq proteins[95–97]. Intriguingly, OX/HCRT differentially regulates firing of projection-specified populations of VTA DA neurons, and notably activates those projecting to the Nac but not those projecting to the amygdala[73]. This difference could be due, in part, to differential expression of OX/HCRT receptors on VTA neurons projecting to the Nac vs. the amygdala, though this has yet to be shown definitively. Going forward, it will be instructive to determine if OX/HCRT receptor expression in the VTA can be used to distinguish DA subpopulations and their functions.

1.4.2 ***Neurotensin***

Many neurons expressing the neuropeptide neurotensin (Nts) provide input to the VTA, with the largest populations originating from the medial preoptic area, Nac and the LHA[98]. Interestingly, at least some LHA Nts-expressing neurons are regulated by leptin; approximately 30% of Nts neurons co-express the long form of the leptin receptor (LepRb)[66]. Given that leptin itself suppresses feeding, leptin acting on LHA Nts-containing neurons might promote anorexic effects, perhaps via Nts itself. While this has yet to explored explicitly, activating LHA Nts-expressing neurons does increase Nts release into the VTA[99,100], and pharmacologic Nts treatment into the VTA has been shown to reduce feeding[101,102]. Moreover, pharmacologic administration of Nts in the VTA potentiates excitatory synaptic transmission and increases the firing rate of DA neurons, which in turn promotes the release of DA in the Nac[103,104]. This leads to feeding restriction, increased locomotor activity and ultimately weight loss in obese rodents[101,105,106]. In combination, these data confirm that Nts modulates VTA DA neurons and can exert anorectic effects. It is notable that both Nts and OX/HCRT are reported to activate VTA DA neurons but exert opposing effects on feeding. One possible hypothesis to explain this conundrum is that these neuropeptides might engage separate subsets of VTA DA neurons that differentially modify feeding. If true, expression of receptors for OX/HCRT vs. Nts could be a means of discerning which VTA neurons respond to these neuropeptides, and whether they comprise separate subpopulations. This hypothesis has yet to be directly explored. However, comparing the distributions of OX/HCRT and Nts receptors in the VTA will be vital to assess how

each of these neuropeptides can promote activation of VTA DA neurons but yield completely different control of behavior.

The VTA can directly respond to Nts since it contains both of the signaling forms of the neurotensin receptors, neurotensin receptor-1 and -2 (NtsR1 and NtsR2)[55,107,108]. Intriguingly, Nts action via NtsR2 is not a direct modulator of VTA DA neurons, since NtsR2 is almost exclusively expressed in VTA astrocytes[109]. By contrast, NtsR1 is the predominant receptor isoform expressed by VTA DA neurons[55], and is vital for Nts-mediated DA release into the Nac[100]. Moreover, only a subset of VTA DA neurons express NtsR1, and these neurons preferentially project to the Nac and not to the PFC[55]. Thus, NtsR1 represents a molecular marker of primarily mesolimbic, not mesocortical VTA DA neurons, but the specific role of these VTA NtsR1 neurons remains incompletely understood. Loss of NtsR1 increases feeding but also further elevates energy expenditure (primarily through an increase in physical activity), and this energy imbalance promotes leanness[109]. Furthermore, intact NtsR1 expression is required for LHA Nts-LepRb neurons to restrain feeding, indicating the functional integration of leptin and Nts/NtsR-1 action[71]. At least some of these feeding restraint effects are mediated via the LHA Nts neurons that project densely to the VTA[99,100], and depends on NtsR1[99]. Taken together, these data indicate that Nts input to the VTA can promote weight loss behaviors, at least in part, due to regulation of NtsR1-expressing DA neurons. It yet remains unclear how the VTA NtsR1 neurons specifically mediate anorectic signals, or if they are sufficient to promote weight loss behaviors, which warrants examination in the future.

1.4.3 ***Galanin***

LHA galanin neurons partially overlap with LepRb-expressing neurons or Nts neurons[110–113]. Since some LepRb and Nts neurons project to the VTA it was long reasoned that galanin was likely released into the VTA as well. Intriguingly, recent evidence suggests that LHA galanin neurons are one of the few LHA populations that do not engage the VTA directly, and instead project locally within the LHA to regulate OX/HCRT neurons that express the galanin receptor. LHA galanin neurons do impact feeding via this local circuit, specifically modifying nutrient preference (sucrose > fat)[111]. Mice that lack the LepRb from their LHA galanin expressing neurons show an increase in OX/HCRT neuronal activation[111], which suggests that LHA galanin-LepRb expressing neurons inhibit OX/HCRT neurons under baseline conditions. Importantly, it remains possible that LHA galanin neurons can indirectly impact activation of VTA DA neurons via modulating OX/HCRT neurons that project to the VTA. On the other hand, activation of LHA Nts neurons, some of which co-express galanin[112], can modestly suppress feeding[99]. Since many LHA Nts neurons project to the VTA[98], these data suggest that there must be a population of Nts-only neurons in the LHA that project to the VTA, in addition to an LHA subset that co-expresses Nts and galanin and projects locally. It is therefore possible that the LHA Nts-only neurons that project to the VTA mediate Nts-mediated anorectic actions via the NtsR1 expressed on VTA NtsR1-DA neurons.

1.4.4 ***Melanin-Concentrating Hormone***

Melanin-concentrating Hormone (MCH) is a defining neuropeptide of the LHA, as its expression is restricted to this brain region. While some studies have described MCH fibers and mRNA from MCH receptors in the VTA, others show low to no expression of MCH receptors in the VTA[95,114–117]. Although the LHA as a whole provides dense input to the VTA, the widely accepted view is that MCH neurons are a notable exception, and do not significantly project to or directly regulate the VTA[115]. Nevertheless, MCH has well-established connections with other sites that contribute to changing behavior (cerebral cortex, LHA, amygdala, and Nac[118–121]), where it has been implicated in orexigenic intake of foods, as well as in cocaine- and amphetamine-induced reward and reward learning[122,123]. Thus, while MCH is an LHA neuropeptide with important roles in modulating feeding, it does not exert these actions via direct engagement of VTA DA neurons.

1.4.5 ***Corticotropin Releasing Factor***

Corticotropin releasing factor (CRF) is widely expressed throughout the brain, including regions of the forebrain, the VTA and within a subset of LHA neurons that are activated by dehydration anorexia[124–126]. In addition, pharmacologic and endogenous CRF suppress feeding in both lean and obese mice[127]. CRF signals through the CRF receptor-1 and -2 (CRFR1, CRFR2). Not much is known about CRFR2 signaling, but the CRF/CRFR1 system has been repeatedly associated with stress and addiction[128,129]. Accordingly, CRFR1 is expressed in DA neurons of the

VTA and the adjacent substantia nigra pars compacta (SNc)[130]. A recent study revealed that VTA CRFR1 expressing DA neurons primarily project to the Nac core, rather than the Nac shell, and their activation is important for associating stimuli and behaviors more so than augmenting the wanting of those stimuli[44]. While feeding was not specifically studied, it may be that VTA CRFR1 neurons play a role in learned feeding behaviors, and this will merit further attention. Going forward it will be important to investigate if LHA-derived CRF signaling modulates feeding via the VTA CRFR1 neurons, and what the role of the CRF neuropeptide is in this process vs. other signals released from LHA CRF neurons.

1.5 SUMMARY AND IMPLICATIONS

VTA DA neurons are now recognized to be heterogenous via multiple criteria, and this opens the door to investigating whether subsets of VTA DA neurons may mediate different aspects of feeding behavior. Given the significant diversity in the types of signals that access the VTA, it seems possible that VTA DA neurons may accordingly tune feeding behavior in response to them. Going forward, "mapping" the populations of VTA DA neurons via their neuropeptide receptor expression could prove instructive, as it might identify subsets that contribute to orexigenic or feeding restraint behaviors. Given that LHA neurons project to, and act in concert with VTA DA neurons to modify behavior, assessing VTA DA neurons that express receptors for LHA-released signals might suggest which neurons modulate feeding, and how they do so. However, it is likely that subsets of VTA DA neurons will not express a single neuropeptide receptor subtype, but in fact, a suite of neuropeptide receptors. Such a system could enable

VTA DA neurons to flexibly respond to different cues and from different inputs, not all of which may be active at the same time. Thus, it remains possible that there could be subsets of DA neurons that primarily mediate orexigenic responses vs. those that constrain feeding or other rewards and mediate these responses via a collection of expressed neuropeptide receptors. There remains much to learn, however, regarding the mechanisms by which neuropeptides modulate the VTA and feeding. For example, neuropeptides from the LHA (and elsewhere) may change the balance of tonic vs. burst-firing of DA neurons, the latter of which is associated with changing goal-mediated behavior. Thus, in the future it will be important to characterize how specific neuropeptides, or combinations of them, impact the firing pattern of DA neurons. It is also likely that neuropeptides may modulate other VTA neuronal populations (e.g., GABA or glutamate containing neurons) or glial populations, which can in turn indirectly modify the activity of local DA neurons or other external targets. While we have focused on how LHA neuropeptides can directly engage DA neurons that express their cognate receptors, neuropeptide modulation of other VTA cells could have powerful effects on feeding behavior and warrant further study. In any case, going forward it will be important to determine if neuropeptide receptors can be used to molecularly distinguish the projection and functionally distinct populations of DA neurons, or other VTA cell types that impact feeding. These studies would not only advance understanding of the basic biology regulating energy balance but could also suggest how to design molecular-based tools to dissect the function of specific VTA cell subsets.

1.6 GOALS OF THE DISSERTATION

Obesity is caused by excess food intake along with decreased physical activity, and it increases the risk of developing type-2 diabetes and other chronic conditions that diminish lifespan[5,6]. Distinct subsets of dopamine (DA) neurons in the ventral tegmental area (VTA) have been implicated in the pathogenesis and potential treatment of obesity. However, the lack of molecular markers to distinguish VTA DA populations has prevented identification of the precise DA neurons to optimally modulate feeding and locomotor activity to support weight loss. Thus, there is a critical need to molecularly define the precise VTA DA neurons that promote weight loss behaviors, which might be therapeutic targets for obesity.

We have identified a specific subset of VTA DA neurons that express neurotensin receptor-1 (NtsR1), henceforth called "VTA NtsR1 neurons". This discovery is important because it reveals NtsR1 as a molecular marker that can be used to distinguish VTA DA neurons and suggests a functional role for NtsR1 signaling in energy balance. Indeed, infusion of the neuropeptide neurotensin (Nts) specifically into the VTA restrains feeding and promotes locomotor activity, thus promoting dual behaviors to support weight loss[99,101,106]. NtsR1 is required for Nts-mediated suppression of feeding[55,99], and whole-body NtsR1 knock-out mice exhibit increased intake of palatable foods that promote weight gain[71]. Furthermore, activating neurons that project to and release Nts into the VTA promotes energy expenditure and weight loss, presumably via activation of VTA NtsR1 neurons[99,131]. My data suggest that activation of the specific population of VTA DA neurons that express NtsR1 promotes weight loss behaviors

that lead to weight loss particularly in obese mice[132]. Collectively, these data informed the central hypothesis of my PhD thesis: **VTA NtsR1 neurons are sufficient and necessary for weight loss behavior dependent on signaling via NtsR1**. To examine this hypothesis, I used newly generated reagents that uniquely allowed me to modulate the *in vivo* function of VTA NtsR1 neurons, or their expression of NtsR1. These allowed me to complete the following goals:

1. **Establish whether specifically activating VTA NtsR1 neurons promotes dual weight loss behaviors without negatively altering the cardiovascular system (Chapter 2).**

Hypotheses: 1) The suppression of feeding and promotion of physical activity of Nts in the VTA is due to activation of VTA NtsR1 neurons. 2) The vasodepression and hypothermia observed with systemic or hindbrain Nts treatment is not due to activation of VTA only NtsR1 neurons.

Methods: I used cre-mediated activatory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to selectively activate VTA NtsR1 neurons in normal weight and obese *NtsR1*^{Cre} mice. This method helped reveal if these neurons modify feeding and activity to prevent weight gain and/or promote weight loss. Additionally, I used radiotelemeters and core body temperature measurements to assess whether activating VTA NtsR1 neurons lowered mean arterial pressure and body temperature. These data revealed whether activating VTA NtsR1 neurons was sufficient to promote dual weight loss behaviors without causing adverse cardiorespiratory effects, and if they may be rational biologic targets to treat obesity.

2. Determine the necessity for VTA NtsR1 in energy balance during development and adulthood (Chapter 3).

Hypotheses: 1) Developmental loss NtsR1 from DA neurons will impair feeding, locomotor activity and DA dependent motivated responses. 2) VTA NtsR1 is the specific mediator of Nts in VTA effects (reduced feeding and increased physical activity) and deleting NtsR1 specifically from VTA DA neurons will promote weight gain by increasing food intake and decreasing locomotor activity.

Methods: I used newly generated *NtsR1*$^{flox/flox}$ and 1) crossed them to *DAT*^{Cre} mice to generate mice with developmental deletion of NtsR1 from all DAT expressing neurons (DATR1^{Null}), and 2) injected cre directly into the VTA to conditionally delete NtsR1 in adulthood (VTAR1^{Null}). These data revealed whether NtsR1 in DAT neurons was needed for development of normal weight and whether specific VTA NtsR1 deletion in adulthood is needed for weight maintenance/weight loss in normal and obese mice. Studying these mice will establish the specific role of VTA NtsR1 for energy balance.

By completing these goals, I revealed the importance of the molecularly defined population of VTA NtsR1 neurons for energy balance. Specifically activating VTA NtsR1 neurons is sufficient to support weight loss and deleting VTA NtsR1 in adulthood promotes weight maintenance. Activating VTA NtsR1 neurons promotes weight loss by recapitulating the dual weight loss behaviors observed with intra VTA Nts treatment: a decrease in food intake and a

decrease in physical activity. Moreover, these effects are not accompanied by vasodrepression, hypothermia, or DA-mediated anxiety behaviors. Developmentally deleting NtsR1 from DAT neurons did not impair feeding, locomotor activity or DA dependent behaviors, and deleting NtsR1 specifically from VTA DA neurons in adulthood revealed that the receptor itself is important for weight loss behaviors and weight maintenance, but it is not the only factor mediating these effects.

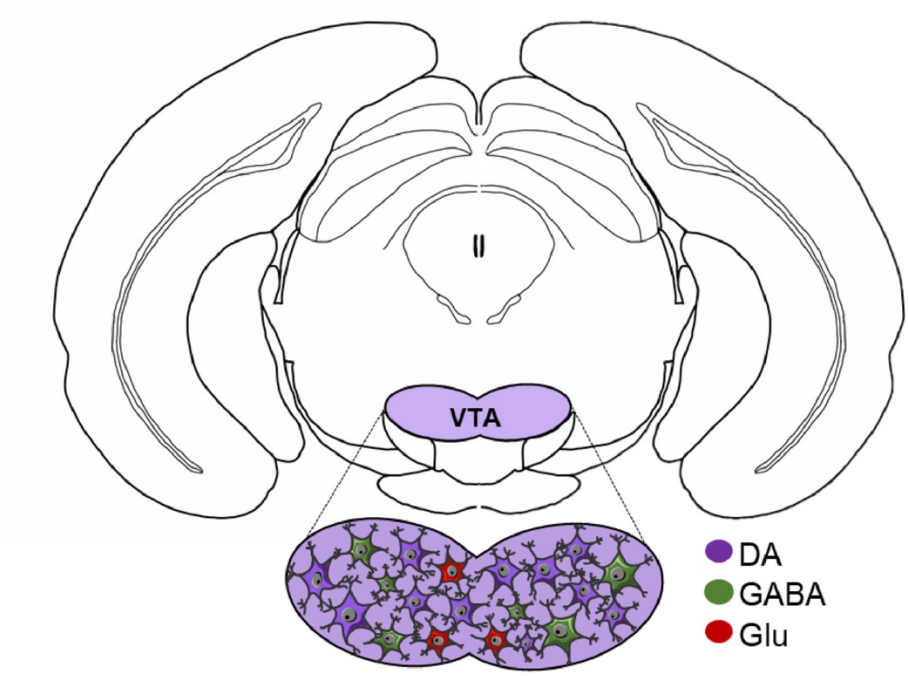

Figure 1.1 Neuronal Composition of the VTA Illustrated on a Coronal Section of a Mouse Brain. The VTA is primarily composed of DA neurons, followed by GABA and glutamate (Glu)-containing neurons.

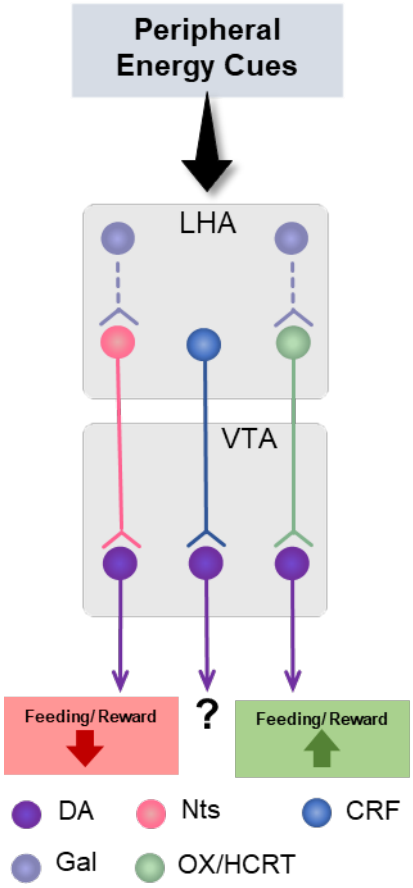

Figure 1.2 Summary of LHA Neuropeptidergic Input to VTA DA Neurons. VTA DA neurons are directly innervated by neuropeptide-containing neurons from the LHA that can decrease or promote feeding and reward behaviors. From the LHA, projecting neurons include neurotensin (Nts), corticortropin releasing factor (CRF) and orexin/hypocretin (OX/HCRT). Although CRF neurons also project to VTA DA neurons, their contribution to feeding/reward behavior is still not clear. Galanin (Gal) does not directly innervate DA neurons but rather projects to OX/HCRT neurons within the LHA that may project to the VTA.

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CHAPTER 2. ACTIVATION OF VENTRAL TEGMENTAL AREA NEUROTENSIN RECEPTOR-1 NEURONS PROMOTES WEIGHT LOSS

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ABSTRACT

Dopamine (DA) neurons in the ventral tegmental area (VTA) modulate physical activity and feeding behaviors that are disrupted in obesity. Yet, the heterogeneity of VTA DA neurons has hindered determination of which ones might be leveraged to support weight loss. We hypothesized that increased activity in the subset of VTA DA neurons expressing neurotensin receptor-1 (NtsR1) might promote weight loss behaviors. To test this, we used Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to activate VTA NtsR1 neurons in normal weight and diet-induced obese mice. Acute activation of VTA NtsR1 neurons (24hr) significantly decreased body weight in normal weight and obese mice by reducing food intake and increasing physical activity. Moreover, daily activation of VTA NtsR1 neurons in obese mice sustained weight loss over 7 days. Activating VTA NtsR1 neurons also suppressed how much mice worked to obtain sucrose rewards, even when there was high motivation to consume. However, VTA NtsR1 neural activation was not reinforcing, nor did it invoke liabilities associated with whole-body NtsR1 agonism such as anxiety, vasodepressor response or hypothermia. Activating VTA NtsR1 neurons therefore promotes dual behaviors that support weight loss without causing adverse effects and is worth further exploration for managing obesity.

2.1 INTRODUCTION

More than one-third of U.S. adults are obese and at increased risk to develop type-2 diabetes[1,2]. Complex interactions between genetic factors and obesogenic environment can increase susceptibility for weight gain[3,4]. However, the most recognized cause for obesity is overconsumption of calorie-dense foods combined with a sedentary lifestyle and reduced energy expenditure. Indeed, since ~1970, the energy cost of work has decreased in the United States as the quantity and energy density of foods have increased, and so has the incidence of overweight and obesity[5–7]. Bariatric surgery is increasingly being used to treat severe obesity but there is a debate about its long-term benefit, as the surgery can induce a myriad of complications[8,9]. Current non-surgical treatments for obesity, including lifestyle modification and pharmacotherapy, provide limited long-term weight loss[10]. Individuals who lose weight experience compensatory increases in appetite and diminished metabolic rate, and as a result most regain weight[11,12]. Understanding how the brain coordinates feeding, physical activity and energy expenditure may be helpful to understand the basic biology of energy balance, and to identify mechanisms to support sustained weight loss.

Dopamine (DA) neurons in the ventral tegmental area (VTA) are essential modulators of feeding and locomotor activity[13,14] and might be useful targets to promote behaviors to favor weight loss. However, VTA DA neurons are highly heterogeneous, differing in connectivity, gene expression and how they modulate behavior, ranging from positive and negative reinforcement, decision making and stimulus salience or aversion [15–19]. This heterogeneity has

hindered determination of which specific DA neurons might be leveraged to support weight loss. Subsets of VTA DA neurons have begun to be distinguished via their expression of neuropeptide receptors[15,17,20], which hints at their potential contributions to energy balance, given that neuropeptides may be orexigenic or anorectic. One subset of VTA DA neurons co-expresses the $G_{\alpha q}$ protein-coupled neurotensin receptor-1 (NtsR1), which binds the neuropeptide Neurotensin (Nts)[21]. Nts exerts diverse, site-dependent regulation of pain, sleep, locomotor activity and ingestive behavior, but within the VTA Nts suppresses feeding and promotes locomotor activity - dual behaviors that could support weight loss[22–25]. The cellular mediators of Nts effects in the VTA remain unclear, however, because this region contains NtsR1 and DA co-expressing neurons as well as astrocytes expressing neurotensin receptor-2 (NtsR2)[20]. Since previous pharmacological reagents were not truly selective for NtsR1 vs. NtsR2, they have not been sufficient to disentangle their contributions to behavior[26,27]. Likewise, mice constitutively lacking NtsR1 or NtsR2 likely suffer from developmental compensation, and may not reflect the physiology of the normal, adult NtsR1 and NtsR2 systems[20]. Thus, although Nts exerts beneficial weight loss effects via the VTA, the cells and receptors mediating these behaviors are still in question.

There is rationale to home in on NtsR1 in Nts-mediated regulation of body weight because mice genetically lacking NtsR1 overconsume palatable food that promotes weight gain [28–31]. Nts activates VTA DA neurons and promotes DA release [32–34]. Intriguingly, at least in mice, the subset of all VTA DA neurons that co-express NtsR1 preferentially projects to the nucleus accumbens (Nac), where DA release can modulate ingestive behavior [20]. These data suggest the

possibility that activating the VTA NtsR1 subset of DA neurons mediates specific functions amongst all DA neurons, and possibly the Nts-mediated effects in the VTA that evoke weight loss. If true, augmenting NtsR1 signaling could have therapeutic potential to reduce body weight. However, initial interest in clinically modulating NtsR1 waned because systemic or brain-wide treatment with Nts or first-generation NtsR1 agonists suppress feeding but also causes dangerous vasodepression and hypothermia[35–38]. Recent work shows that activating site-specific Nts or NtsR1-expressing neurons mediates specific physiology without invoking these adverse effects[30,39–41]. Thus, here we examined whether selective activation of VTA NtsR1 neurons could promote weight loss behaviors without causing adverse thermoregulatory or cardiorespiratory effects. To test this hypothesis, we expressed Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in VTA NtsR1 neurons of normal weight and diet-induced obese *NtsR1^{Cre}* mice, which permitted *in vivo* activation of VTA NtsR1 neurons. Our data suggest that activating VTA NtsR1 neurons recapitulates Nts-mediated behaviors useful for weight loss without undesirable side effects.

2.2 RESEARCH DESIGN AND METHODS

2.2.1 ***Mice***

All mice used herein were bred and housed under a 12hr light/12hr dark cycle, and were cared for by Campus Animal Resources (CAR). We studied male and female heterozygous *NtsR1^{cre}* mice (*NtsR1$^{cre/+}$)* on the C57/Bl6J background, Jackson Stock #033365) and littermate

controls (*NtsR1*$^{+/+}$, referred to as wild type, *Wt)*. At 4 weeks of age the study mice were individually housed with *ad libitum* access to water and either chow (Harlan Teklad #7913) or 45% high-fat diet (HFD, Research Diets D12451) for the duration of experiments, unless otherwise specified. Multiple cohorts of *NtsR1*^{cre} and *Wt* mice were generated and the mice were tested via multiple tests, as described below. Cohorts were staggered to control for any seasonal effects. Since we did not observe any metabolic or behavioral differences between males and females in our tests, both sexes were pooled. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health.

2.2.2 ***Surgery***

NtsR1^{Cre} mice and *Wt* littermates (8-15 weeks) were anesthetized (isofluorane/oxygen mixture, 2-4%) and given analgesic (Meloxicam, 5mg/kg), prior to bilateral stereotaxic injection of AAV2-hSyn-DIO-hM3D(Gq)-mCherry (UNC Vector core/Addgene) into the VTA (100 nL per side, A/P: –3.2, M/L: ±0.48, D/V: –4.65) per the mouse brain atlas of Paxinos and Franklin [42]. Mice were allowed to recover for at least 2 weeks prior to metabolic and behavioral testing. Brains were examined via posthoc immunostaining for mCherry and TH (see immunostaining method below) to verify targeting and DREADDq-mCherry expression in the VTA. *NtsR1*^{Cre} mice were only included in the final data if mCherry-expressing soma were confined to the VTA, and any mice with mCherry soma beyond the VTA were excluded. Mice that were bilaterally

targeted in the VTA with DREADDq responded to CNO treatment with robust (>500) wheel rotations, revealing this as a reliable indicator of VTA NtsR1 targeting. Mice with robust unilateral targeting exhibited similar CNO-induced wheel rotations and were also included in the final study data (10% of cases).

2.2.3 ***Treatment***

Vehicle (VEH, PBS) or the DREADD-ligand clozapine-N-oxide (CNO, 0.3 mg/kg) were administered via *i.p.* injection. CNO (C0832, Sigma) was dissolved in 10% β-cyclodextrin (C0926, Sigma) in sterile PBS to make 20X CNO stock aliquots (1.2mg/mL), which were diluted with sterile PBS to make the 1X CNO working stock (60μg/mL). We then administered 5μL of 1X CNO per g of body weight. Unless specified otherwise, mice were treated twice a day, 1-2hr after onset of light cycle and 1-2hr before dark cycle, via a crossover design. Thus, all mice received both VEH and CNO with at least 3 days between treatment switch.

2.2.4 ***Metabolic Analysis***

Mice were analyzed in PhenoMaster metabolic cages (TSE Systems) 2-3 weeks after AAV2-hSyn-DIO-hM3D(Gq)-mCherry injection. Metabolic cages continuously monitored food and water intake, locomotor activity, wheel running, and metabolic parameters (VO2, respiratory exchange ratio [RER], and energy expenditure). Mice acclimated in cages for 48hr prior to receiving daily treatments during the light and dark cycles. Mice received sham

injections (day 3), followed by VEH (day 4), CNO (day 5) and then were left in the metabolic cages for a 24hr washout period. Weight was measured just prior to the light cycle injection each day and at the end of the washout period. Ambient temperature was maintained at 20–23°C and airflow rate was adjusted to maintain an oxygen differential around 0.3% at resting conditions.

2.2.5 ***Operant Testing***

Training Chow-fed mice: Mice were trained to nose-poke for unflavored 20 mg sucrose pellets (TestDiet 1811555) in operant-responding chambers (Med Associates) as previously described [43,44]. *Chow experiments*: mice were food restricted to 85% of their body weight during FR1 training sessions, which occurred over 10-16 consecutive days. Each FR1 training session was terminated after 1hr or when the mouse had earned 50 rewards. Once mice achieved 75% response accuracy with ≥20 rewards earned on 3 consecutive days of FR1 training, they were switched to *ad libitum* food and trained on an FR5 schedule for 3 consecutive days. Mice that failed to reach FR1 criteria after 16 days were removed from the study. *Training HFD-fed mice:* We and others have found that 85% HFD caloric restriction does not motivate diet-induced obese mice to acquire FR1 criteria, so we modified the conditions to encourage these mice to learn the operant task. After their first FR1 session the HFD mice were weighed and then switched to a restricted chow diet, so as to maintain them at 80% of their starting body weight throughout FR1 training. HFD mice that did not achieve 75% response accuracy with ≥20 rewards earned on 3 consecutive days were removed from the study. FR1

testing was conducted for a maximum of 14-16 consecutive days, at which point any mice failing to meet the above criteria were removed from the study. Mice achieving FR1 criteria proceeded were then trained on an FR5 schedule for 3 consecutive days. *Progressive Ratio (PR) Testing for All Mice:* After FR5 testing mice were subject to a progressive ratio (PR) schedule where PR=[$5e^{(R*0.2)}$]-5 with R=number of food rewards earned+1. The PR breakpoint was recorded as the highest ratio completed for each 1 hr test session. Mice were tested until they achieved stable PR, defined as <10% variation in rewards earned over 3 consecutive sessions. Next, mice were treated with VEH or CNO (0.3 mg/kg, *i.p.*) 30mins before PR testing. To determine if hunger altered responding, mice were fasted overnight, then were treated with VEH or CNO and tested on the PR schedule. Lastly, to determine if satiety modified responding, mice received 3 g of sucrose pellets in their home cage overnight then were PR-tested the next morning.

2.2.6 ***Sucrose Preference***

Mice were studied in home cages with *ad libitum* access to two bottles containing liquid. For the first 48 hr both bottles contained water, so as to acclimatize mice to the two-bottle arrangement. The position of the bottles was switched every 24hr to control for place preference effects. Place preference during pretesting is cause for exclusion, but did not occur in our study so no mice were excluded. Next, one of the bottles was replaced with 1% sucrose (Sigma) so that mice had a choice of water or sucrose. All mice received both VEH and CNO injections (2X day) in a crossover design. Mice were given at least 24hr before repeating the

experiment with the alternate treatment (e.g., 2 bottles of water for 48hr followed by water vs 1% sucrose for 48hr while being treated). Body and food were weighted every 12hr during the experiment.

2.2.7 ***Conditioned Place Preference (CPP)***

CPP (San Diego Instruments) boxes had two distinguishable chambers with different visual and tactile cues (gray wall and smooth floor or striped wall and rough floor) separated by a small center chamber [30]. Mice had a 15-minute pretest on day 1, during which they were allowed to roam freely between chambers. After pretest data were collected, we assigned which chamber was paired with either VEH or CNO injection with an unbiased, counterbalanced strategy so that approximately half the mice received CNO pairing in the preferred side and half received CNO in the non-preferred side. Mice then underwent conditioning on days 2-5, where each morning they received an injection of VEH and were placed in the VEH-paired side for 30 minutes. After the morning session, mice were returned to their housing environment and 4 hours later they were injected with CNO and were placed in the CNO-paired side for 30 minutes. VEH and CNO injections occurred 5 minutes before mouse placement in the corresponding chamber. Posttest was conducted on day 6, where mice were untreated and were allowed to roam freely amongst both chambers for 15 minutes. The time and locomotor activity in each side of the box was detected as laser beam breaks using manufacturer's software.

2.2.8 ***Chronic Activation***

In home cages, HFD-fed mice received twice-daily treatments with either VEH or CNO for 7 days. Body weight and food intake were measured during the light cycle, and on days 1, 5 and 7 mice were placed in open field boxes 2-4 hours after receiving VEH or CNO. Locomotor activity was measured for 30 min using a digital CCD camera and video-tracking software (Clever Sys)[45]. At least 7 days passed before repeating the experiment with the opposite treatment.

2.2.9 ***Nestlet Shredding***

Mice were treated with VEH or CNO in their home cages during the light cycle. After 30 minutes, bedding, food and water were removed, and a pre-weighed, new cotton nestlet was added to the cage. After 90 minutes, intact remnants of the nestlet were removed from the cage, air dried overnight and weighed the following day.

2.2.10 ***Marble Burying***

Mice received VEH or CNO in home cages during the light cycle. 30 minutes later they were placed in the middle of a cage with 10 evenly dispersed marbles on top of the bedding. After 30 minutes mice were returned to their home cage. Photos taken before and after the

test were used to determine the number and percentage of marbles buried (e.g., if 2/3 of the marble was covered).

2.2.11 ***Temperature***

Core-body temperature was measured with a rodent rectal thermometer (BIO TK9882) immediately after VEH or CNO treatment, then every 30 minutes up to 120 minutes and for a final measure at 180 minutes. A minimum of 3 days of rest were given before repeating the experiment with opposite treatment.

2.2.12 ***Telemetry***

Under isofluorane/oxygen mixture (2-4%), mice were implanted with subcutaneous PA-C10 radiotelemetry transmitters (DSI, Harvard Bioscience), attached to a catheter inserted into the abdominal aorta via the femoral artery [46]. Transmitters measured mean arterial pressure, activity, heart rate, and systolic and diastolic blood pressure every 10 minutes throughout the experiment. After 3 days of surgical recovery, mice received twice-daily sham injections (day 4) followed by VEH or CNO via crossover design (days 5 and 6).

2.2.13 ***Perfusion and Immunofluorescence***

Mice that did not have telemeters implanted received a lethal *i.p.* dose of pentobarbital (Fatal Plus, Vortech) 90 minutes after VEH or CNO treatment, followed by transcardial perfusion with 0.2M PBS (pH 7.4) and then 10% neutral-buffered formalin (Fisher Scientific). Brains were removed, postfixed in 10% formalin overnight at 4°C and dehydrated with 30% sucrose in PBS for 2-3 days. Mice that did have a telemeter implanted also received a lethal *i.p.* dose of pentobarbital 90 minutes after VEH or CNO treatment, but their brain was removed and postfixed, as formalin perfusion would damage telemeters. Brains were then coronally sectioned (30µm) for immunofluorescence, as per [20]. Sections were exposed to primary antibodies including rabbit-dsRed/mCherry (1:1000, Clontech, AB_10013483), chicken-GFP (1:2000, Abcam, ab13970), mouse anti-TH (1:1000, Millipore, AB_2201528) and goat-cFOS (1:500, Santa Cruz Biotechnology, sc-52-G), followed by species-specific secondary antibodies conjugated to Alexa-568 (1:200, LifeTech, AB_2534017) or Alexa-488 (1:200, Jackson ImmunoResearch, 703-545-155). Sections mounted onto slides were analyzed via an Olympus BX53 fluorescence microscope with FITC and Texas Red filters. Images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera and analyzed using Photoshop (Adobe). Masks applied in Photoshop to enhance brightness and/or contrast were applied uniformly to all samples.

2.2.14 ***Cell Counting***

Two representative levels of the VTA (approximately Bregma -3.38 and -2.98) were chosen for counting, and a 20x image of the right and left VTA hemisphere was collected from each. Images were then coded with non-descriptive names and a blinded investigator used Photoshop to count the number of magenta and green labeled cells in each image. For mCherry and TH immunostaining, a cell was considered co-labeled if both labels overlapped in the whole soma. For mCherry and cFos immunostaining, a cell was counted as co-labeled if the nucleus contained cFos and was surrounded by mCherry in the soma. The number of co-localized and singly labeled cells were summed from the two sections of each brain and used to calculate the percentage of labeled cells. Graphs depict the average percentage of cells ± SEM.

2.2.15 ***Statistics***

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

2.3 RESULTS

2.3.1 VTA NtsR1 Neurons are a Subset of all DA Neurons that Can be Activated with DREADDs

NtsR1^{cre} mice were injected in the VTA with cre-dependent AAV2-hSyn-DIO-hM3D(Gq)-mCherry to express hM3Dq-mCherry (excitatory DREADD) in NtsR1 neurons (Figure 1A). After VEH or CNO treatment (90 min), brains were analyzed for mCherry (hM3Dq-mCherry expressing neurons, purple) and tyrosine hydroxylase (TH, a marker of DA neurons, green) via immunofluorescence (Figure 1B). ~70% of VTA TH neurons co-expressed NtsR1 (Figure 1A - white arrows and Figure 1C), consistent with previous reports [20,43]. Moreover, CNO significantly increased the proportion of mCherry neurons containing cFOS (a marker of depolarization) over VEH treatment (Figure 1D-F, $p<0.0001$). These data confirm that DREADDs can be used to activate VTA NtsR1 neurons.

2.3.2 *Activation of VTA NtsR1 Neurons Promotes Weight Loss Behaviors in Normal Weight Mice*

To determine how activating VTA NtsR1 neurons impacts energy balance, we treated normal weight, chow-fed *NtsR1*^{Cre} mice expressing VTA NtsR1-hM3Dq-mCherry with VEH or CNO while in metabolic chambers (Figure 2A,B). CNO-mediated activation of VTA NtsR1 neurons decreased body weight in these mice over 24 hrs (Figure 2C). Activating VTA NtsR1

neurons modestly decreased food and water intake, primarily during the dark cycle (Figure 2D,E; y axis asterisks indicate overall treatment effect between VEH and CNO). VTA NtsR1 neuronal activation also increased overall ambulatory locomotor activity and wheel rotations during the light and dark cycles (Figure 2F,G; y axis asterisks indicate overall treatment effect between VEH and CNO, while asterisks within the graphs indicate significant differences at specific time points). In addition, activating VTA NtsR1 neurons in chow fed *NtsR1*^{Cre} mice increased energy expenditure (Figure 2H) and decreased RER (Figure 2I), which respectively indicate increased calories/hour burned and increased use of fat as an energy substrate (although not outside normal range, 0.7-1). The change in RER is not surprising here, as decreased RER can result from decreased food intake. After CNO treatment, mice were kept in TSE cages for 24 hours (washout day, no injection given), but there were no differences between VEH and washout days (Figure 2.9A-F). Neither did we observe any differences between chow-fed *Wt* littermates analyzed via the same paradigm (Figure 2.10A-H), confirming that changes were attributed to CNO-mediated activation of VTA NtsR1 neurons. These data support that activation of VTA NtsR1 neurons promotes weight loss in normal weight *NtsR1*^{Cre} mice.

2.3.3 ***Activating VTA NtsR1 Neurons Suppresses DA-Dependent Palatable Food Consumption in Normal Weight Mice***

Since DA neurons modify goal directed behaviors to obtain rewards[47] we examined how the subset of DA neurons expressing NtsR1 impacts operant responding for sucrose pellets (a

test of DA-dependent willingness to work for food rewards). Mice were tested during three motivational states: baseline, fasted, and sucrose pre-fed (sated) (Figure 3A,B). Activation of VTA NtsR1 neurons did not disrupt anticipatory behavior, confirmed by the similar number of magazine entries after VEH and CNO treatment (Figure 1C). Although CNO-mediated activation of VTA NtsR1 neurons decreased the total number of all nose pokes mice performed (Figure 2.11) it did not alter the percentage of correct nose pokes (Figure 3D); this indicates that mice performed the task less, but when they did, they were able to perform it correctly. However, CNO-mediate activation of VTA NtsR1 neurons reduced the number of sucrose pellets mice obtained and their PR breakpoint (willingness to work for sucrose) across all motivational states (Figure 3E,F). Interestingly, when VTA NtsR1 neurons were activated, chow fed *NtsR1*^{Cre} mice ate 90-100% of the sucrose pellets obtained during baseline and fasted conditions, but pre-fed mice only ate ~40% of the sucrose pellets (Figure 3G). Thus, in a sated state, activating VTA NtsR1 neurons not only reduced motivation to obtain palatable rewards, it also suppressed consumption. Conversely, CNO-treatment had no effect on behavior of chow-fed *Wt* littermates (Figure 2.12). Notably, activation of VTA NtsR1 neurons did not restrain preference for freely available 1% sucrose solution over water, a test measuring hedonic value (Figure 2.13). Overall, these results suggest that VTA NtsR1 activation suppresses intake primarily by reducing the willingness to work for food.

2.3.4 *Acute Activation of VTA NtsR1 Neurons Promotes Weight Loss Behaviors in Obese Mice*

To assess the potential of VTA NtsR1 neurons to treat obesity we DREADD-activated VTA NtsR1 neurons in diet-induced-obese *NtsR1^{Cre}* mice. Obese *NtsR1^{Cre}* mice expressing hM3Dq-mCherry in VTA NtsR1 neurons were analyzed in metabolic chambers with *ad libitum* palatable high fat diet (HFD) while receiving twice daily VEH or CNO treatments (Figure 4A,B). CNO-mediated activation of VTA NtsR1 neurons decreased body weight of obese *NtsR1^{Cre}* mice over 24 hrs (Figure 4C). Concomitantly, CNO-activation of VTA NtsR1 neurons in these mice decreased HFD and water intake primarily during the dark cycle, when mice consume most food and water. Yet, activation of VTA NtsR1 neurons increased ambulatory locomotor activity and wheel running during both the light and dark cycles (Figure 4D-G, y axis asterisks indicate overall treatment effect between VEH and CNO). Activation of VTA NtsR1 neurons also increased energy expenditure (Figure 4H) and both increased (light cycle) and decreased (dark cycle) RER (Figure 4I; in addition to significant differences in overall treatment effects, asterisks within graphs indicate significant differences at specific time points). These effects were absent in obese *Wt* mice treated with CNO (Figure 2.14), albeit a modest increase in dark cycle RER that did not mimic the decreased RER in *NtsR1^{Cre}* mice (Figure 2.14H vs. Figure 4I). After CNO treatment, mice were kept in TSE cages for 24 hours (washout day, no injections given). While the suppression of feeding and drinking was no longer present on washout day, mice still exhibited lower weight, increased activity and wheel rotations during the dark cycle, approximately 24 hrs after the last activation of VTA NtsR1 neurons (Figure 2.15). Overall, these

data indicate that activating VTA NtsR1 neurons alters ingestive and locomotor behaviors that promote and maintain weight loss in obese mice.

2.3.5 *VTA NtsR1 Neuronal Activation in Obese Mice Suppresses DA-Dependent Palatable Food Consumption*

We next used operant responding to test whether VTA NtsR1 neurons modulate DA-dependent responding for palatable food in obese mice (Figure 2.5A,B). Given that activating VTA NtsR1 neurons in obese *NtsR1*^{Cre} mice notably increased locomotor behavior (Figure 2.4F), we considered whether this could have impeded their operant responding. As reflected by the number of magazine entries, CNO-mediated activation of VTA NtsR1 neurons in obese *NtsR1*^{Cre} mice decreased anticipatory behavior in baseline and sucrose pre-fed states, but not in fasted mice (Figure 2.5C). Together, these data suggest that obese mice with activated VTA NtsR1 neurons can coordinate hunger status with anticipatory locomotor behavior, and that augmented locomotor activity did not prevent them from engaging the magazine. While CNO-mediated activation of VTA NtsR1 neurons did decrease the total number of all nose pokes mice performed in the fasted and pre-fed states (Figure 2.16), it did not diminish the percentage of correct nose pokes (Figure 2.5D). Thus, mice were able to execute the operant behavior correctly albeit they elected to do so less when VTA NtsR1 neurons were activated. Accordingly, CNO-mediated VTA NtsR1 neuronal activation decreased the number of pellets earned by obese *NtsR1*^{Cre} mice and their PR breakpoint in all three states (Figure 2.5E,F). Moreover, when their VTA NtsR1 neurons were activated, obese *NtsR1*^{Cre} mice ate most of the

sucrose pellets they obtained during baseline and fasted states, but pre-fed mice only ate ~30% of their earned sucrose rewards (Figure 2.5G). As in normal weight mice, activation of VTA NtsR1 neurons in obese mice had no impact on their preference for a freely available 1% sucrose solution, but did reduce HFD intake and weight (Figure 2.17). Overall, these results suggest that VTA NtsR1 neuronal activation in the obese state does not modify the palatability of food but can reduce goal-directed behaviors to obtain it.

2.3.6 ***VTA NtsR1 Neuronal Activation Promotes Sustained Weight Loss in Obese mice***

Next, we tested whether the acute weight-reducing effect of activating VTA NtsR1 neurons could be sustained. Diet-induced obese *NtsR1^{Cre}* mice expressing hM3Dq-mCherry in VTA NtsR1 neurons were treated twice daily for 7 days with VEH/CNO in their home cage (Figure 2.6A). Chronic VTA NtsR1 neuronal activation in obese *NtsR1^{Cre}* mice decreased body weight, which was sustained for the duration of the treatment period, including after the sham/washout day (Figure 2.6B). Overall HFD intake was suppressed by CNO treatment (Figure 2.6C, asterisks at base of y axis indicate overall treatment effect). However, CNO-mediated feeding restraint was more pronounced during the first 4 days of treatment, after which it was comparable to VEH treatment. To assess locomotor activity, mice were placed in open field boxes during the light cycle on treatment days 2, 6, 8 and sham (Figure 2.6D). CNO-treated obese *NtsR1^{Cre}* mice exhibited increased physical activity that progressively reduced over the experiment, but was indistinguishable from VEH-treatment on sham/washout day (Figure 2.6E). Thus, although the feeding suppression and locomotor enhancing effects of activating VTA

NtsR1 neurons dissipate over time, repeated activation of VTA NtsR1 neurons can induce and sustain weight loss in obese mice.

2.3.7 *VTA NtsR1 Neuron-Mediated Suppression of Feeding is not Due to Aversion or Anxiety*

Aversive stimuli or stress can also suppress feeding, so we investigated whether activation of VTA NtsR1 neurons promotes these conditions. Normal weight and obese *NtsR1^{Cre}* mice expressing hM3Dq-mCherry in VTA NtsR1 neurons were pooled for these tests since there were no significant differences between the diet-defined groups. When tested in conditioned place preference chambers paired with either VEH or CNO, *NtsR1^{Cre}* mice demonstrated neither aversion nor preference for the CNO-activation paired chamber (Figure 2.7A). Activating VTA NtsR1 neurons did not increase anxiety-like behavior as measured via nestlet shredding or marble burying (Figure 2.7B,C). Collectively, these data signify that activating VTA NtsR1 neurons does not suppress feeding secondary to aversion or anxiety.

2.3.8 *Activation of VTA NtsR1 Neurons Does Not Induce Hypothermia or Vasodepression*

Systemic or brain-wide treatment with Nts or NtsR1 agonists lowers blood pressure and causes hypothermia and cyanosis in rats [35–38,48–50], but it is unclear which NtsR1 neurons mediate these adverse effects. We tested whether activation of VTA NtsR1 neurons invokes

vasodepression and/or hypothermia in normal weight and obese *NtsR1*^{Cre} mice (Figure 2.8A). CNO-mediated activation of VTA NtsR1 neurons significantly increased core body temperature in normal weight but not obese, *NtsR1*^{Cre} mice (Figure 2.8B,C), but did not produce the hypothermia observed with systemic NtsR1-agonism. We then implanted telemeters in these mice to measure blood pressure, heart rate and physical activity. CNO-mediated activation of VTA NtsR1 neurons increased mean arterial pressure (Figure 2.8D,E), and heart rate only during the light cycle (Figure 2.18B, C) though it increased locomotor activity during the light and dark cycles (Figure 2.18D, E). Interestingly, ~60-70% of these cardiovascular effects are attributable to the increased locomotor activity in *NtsR1*^{Cre} mice (Figure 2.8F,G and Figure 2.18F, G). Thus, selectively activating VTA NtsR1 neurons does not recapitulate the hypothermia or vasodepression previously described with systemic NtsR1 agonism.

2.4 DISCUSSION

Nts can suppress feeding and increase locomotor activity via the VTA, but prior limitations prevented understanding if these effects are mediated via NtsR1 or NtsR2-expressing cells. Herein we show that activating VTA NtsR1 neurons, a subset of all VTA DA neurons, restrains feeding and drinking, promotes locomotor activity, and invokes weight loss in normal weight and obese mice of both sexes. Activating VTA NtsR1 neurons does not reduce preference for palatable food, but dampens how much mice will work for it, which might be useful to curb caloric intake. Moreover, experimental activation of VTA NtsR1 neurons does not cause the hypothermia, vasodepression or reinforcement effects invoked by global modulation of DA or NtsR1 signaling. Since activating VTA NtsR1 neurons largely recapitulates the effects of

Nts treatment in the VTA, our findings implicate VTA NtsR1 neurons as mediators of Nts-mediated weight loss, and worth exploring as targets to support weight loss without adverse side effects.

Activating all VTA DA neurons produces a range of differential responses, but only some support weight loss[17,51–53], likely due to the heterogeneity of VTA DA neurons and their projections[16,54–58]. We reasoned that certain VTA DA neuronal subsets might be useful to invoke weight loss, and used NtsR1 as a genetic marker to identify and modulate one subset. Importantly, since >90% of VTA NtsR1 neurons co-express TH[43], they are a subset of the large DA population rather than the comparatively smaller GABA or glutamate containing VTA populations. Thus, VTA NtsR1 neuron-mediated effects are likely due to release of DA rather than GABA or glutamate. While VTA NtsR1 neurons comprise a large portion (~70%) of all VTA DA neurons, they are a subset that selectively projects to the Nac[20], which plays an important role in modulating goal-directed behaviors[47,59]. Moreover, given that Nts exerts anorectic actions via the VTA[22,60], we hypothesized that VTA NtsR1 neurons might contribute to this effect. Indeed, activating the VTA NtsR1 neuronal subset restrained feeding, promoted locomotor activity, and supported weight loss, consistent with the effects of Nts administered into the VTA. These effects were observed in both males and females, which is why we pooled data from both sexes. However, given that DA signaling can differ in males and females[61] it is possible that hormonal or environmental context could influence the role of VTA NtsR1-DA neurons, and this should be explored in the future. Indeed, loss of function NtsR1 variants have

been characterized in eating disorders that are more common in females than males[62], suggesting that there may be sex- and context-specific roles for VTA NtsR1 neurons.

A potential caveat of this study is the use of *NtsR1*^{Cre} mice to genetically modulate VTA NtsR1 neurons, and the use of DREADDs to activate them outside of endogenous regulation. It is recognized that introducing IRES Cre after the stop codon (as is the case in *NtsR1*^{Cre} mice) can sometimes influence expression of the upstream coding sequence[63]. Expression of DREADD is thought to be inert, including in DA neurons[53] but some work suggests it could impact neuronal mechanics and dynamics[64]. While we do not have data to suggest that either of these were factors in our current study, they could conceivably lead to differences in baseline behavior of *Wt* and *NtsR1*^{Cre} mice. To control for this possibility we used a within-subjects crossover treatment design, so that *NtsR1*^{Cre} received both VEH and CNO, and could be used as their own controls[64,65]. We treated *Wt* mice to assess any potential off-target effects of CNO, which was particularly important for this study since high doses of CNO have been shown to inhibit DA-mediated locomotor activity[66]. Importantly, the low dose of CNO used in these studies does not alter locomotor activity in *Wt* mice, verifying that it does not exert off-target regulation of the DA system that would confound our studies. Out of an abundance of caution, and in case *Wt* mice may not be identical to *NtsR1*^{Cre} mice, we did not directly intercompare their data. Alternatively, we could have compared *NtsR1*^{Cre} mice injected with cre-inducible DREADDq vs. mCherry, but this study design would prevent the opportunity to discover any potential behavioral differences between *Wt* and *NtsR1*^{Cre} mice.

DREADD-activation of VTA NtsR1 neurons, while artificial, did recapitulate the feeding suppression and locomotor activity induced by Nts or NtsR1 agonists administered into the VTA [22,30,60]. Physiologic release of Nts into the VTA also produces some of these behaviors, suggesting a role for Nts-NtsR1 signaling in mediating the effects. For example, some lateral hypothalamic area neurons express Nts (e.g. LHA Nts neurons) and release it to the VTA and other sites[67]. Activating LHA Nts neurons promotes locomotor activity, restrains feeding and leads to weight loss in normal weight mice, similar to chemogenetically activating VTA NtsR1 neurons[30]. However, while activating VTA NtsR1 neurons sustained weight reduction in obese animals, activating LHA Nts neurons did not. This discrepancy may be due to the net activation of LHA Nts projections to the VTA and other brain areas, which initially invokes water intake that increases body weight followed by weight reducing behaviors[30]. By contrast, activating VTA NtsR1 neurons decreased water intake. One explanation for this effect is that since VTA NtsR1 activation reduces the motivation to obtain food rewards, it could also reduce the motivation to drink, as DA has been associated with the act of drinking and not hydration state[68,69]. Since activating VTA NtsR1 neurons did not promote drinking, LHA Nts neurons may mediate drinking via non-VTA projections. Nts-mediated feeding restraint may be mediated via the VTA, as it was observed after activating LHA Nts neurons[30] and VTA NtsR1 neurons (herein). Circuit-specific activation methods will be necessary to dissect the physiological role of Nts released to the VTA from the LHA vs. other sites. However, our data suggest that activating VTA NtsR1 neurons could be a mechanism to reduce feeding and promote weight loss.

Activation of all VTA DA neurons has been largely characterized as facilitating reward intake. Indeed DREADDq-mediated activation of VTA DA neurons in rats increases cocaine reinstatement but contrarily it decreases free cocaine consumption[53], suggesting that context is important. Likewise, increasing DA signaling has been associated with both increasing and decreasing drug and food intake. Chemogenetic activation of all VTA DA neurons increases responding for sucrose rewards under progressive ratio reinforcement, but psychostimulant drugs that increase DA signaling suppress food intake[51,52], indicating the complexities of DA-mediated behavior. Our studies differ from prior bulk activation of all VTA DA neurons because here we only activated the subset expressing NtsR1 that are known to project to the Nac. Moreover, we purposely used a DREADDq approach to activate VTA NtsR1 neurons because NtsR1 is Gq-coupled receptor. While CNO-mediated activation of DREADDq is not the same as Nts or NtsR1 agonist-mediated activation of NtsR1, we reasoned that it was a reasonable proxy, since Nts and NtsR1 agonists administered into the VTA activate DA neurons expressing the Gq-coupled NtsR1 and suppress food intake[22,70,71]. Likewise, we found that activating VTA NtsR1 neurons reduced feeding and operant responding for palatable food. Because VTA NtsR1 neurons are a subpopulation of all VTA DA neurons that project to the Nac, it is important to consider that their activation could promote DA associated behaviors observed with DA release in the Nac, such as reduction of food intake and hyperlocomotion. It is also worth noting that hyperlocomotion by itself can result in decreased food intake, and we cannot rule out that VTA NtsR1-induced physical activity may contribute to suppressed feeding. However, this elevated locomotor activity did not prevent mice from engaging in anticipatory and operant behaviors, nor from obtaining a freely accessible sucrose solution, suggesting it did not completely impede

feeding. VTA NtsR1 activation lessened the extent to which mice worked to obtain sucrose rewards, but it did not provide an absolute brake on feeding. Rather, VTA NtsR1 neurons may only reduce how much rewards are "wanted" and will be worked for, consistent with the roles of VTA DA neurons in modulating the incentive salience of food and drug rewards[72,73]. Food intake is also influenced by its hedonic valence (e.g., how much it is liked) and is mediated via a DA-independent mechanism. The divergence in wanting vs. liking[74] may explain why activating VTA NtsR1 neurons could not completely prevent operant responding for sucrose; the palatability of the sucrose likely drives some degree of hedonic intake that cannot be mitigated by DA-containing VTA NtsR1 neurons. Indeed, activating VTA NtsR1 neurons did not blunt intake of freely accessible sucrose solution, a test that assesses sucrose's hedonic value. Mechanistically, these data suggest that DA-containing VTA NtsR1 neurons may not modify the opioid signaling systems that encode food "liking" and so cannot completely blunt intake of freely available, palatable foods that promote weight gain. However, because activating VTA NtsR1 neurons in a sated state both restrained the motivation to obtain palatable food and to eat it, these neurons may have promise to prevent overeating and weight gain. Conditioned taste aversion could also account for diminished feeding and can develop after a single exposure to an aversive stimulus. Although the mice in this study went through multiple trials of VTA NtsR1 activation, their food intake did not significantly differ from VEH after during the washout period, arguing against induction of a conditioned taste aversion. (Figure 2.10 & 2.14). However, we cannot exclude that taste aversion might contribute to VTA NtsR1-mediated feeding restraint.

We did, however, evaluate whether activating VTA NtsR1 neurons causes other adverse physiology that would preclude leveraging these neurons to treat disease. Some VTA DA signaling has been implicated in promoting anxiety, aversion or reinforcement that can secondarily reduce feeding behavior[75,76]. However, our battery of tests shows that activating VTA NtsR1 neurons does not invoke these conditions. We also assessed the cardiorespiratory consequence of activating VTA NtsR1 neurons, since the most widely reported effects of systemic treatment with Nts or NtsR1 agonists are hypotension and decreased core body temperature[35–38,48]. While results can differ by tissue and treatment paradigm, in rodents Nts treatment in the central nervous system treatment is well established to elicit hypothermia and a dose related decrease in mean arterial pressure and heart rate[49,50]. These clinical liabilities have diminished enthusiasm for pharmacologically modulating the Nts-NtsR1 system to treat disease. However, the advent of tools to site-specifically modulate Nts and NtsR1 neurons revealed that dedicated Nts/NtsR1 circuits modulate specific physiology, which opened the door for the possibility that only certain circuits mediate cardiorespiratory effects[47,77]. We found that activating VTA NtsR1 neurons promoted weight loss behaviors but did not make mice hypothermic or lower their blood pressure. Moreover, VTA NtsR1 neural activation during the light cycle did not reduce, but rather increased mean arterial pressure and heart rate. Physical activity was also increased during VTA NtsR1 activation and part of the mean arterial pressure and heart rate increase effect (during the light cycle) is due to this increase in physical activity. An increase in mean arterial pressure and heart rate is not surprising here, since during exercise, cardiac output increases more than the total peripheral resistance, which results in an increase in mean arterial pressure[78]. Cardiac output, in turn, is dependent on heart rate; an

increase in heart rate leads to an increase in cardiac output[78]. Since only 60-70% of the variation in mean arterial pressure and heart rate is explained by increased activity, we cannot rule out that activating VTA NtsR1 neurons may drive other physiology that contributes to elevated body temperature and cardiovascular response. Curiously, activating VTA NtsR1 neurons during the dark cycle, when mice are normally alert and active, had no effect on blood pressure or heart rate. However, dark cycle activation of VTA NtsR1 neurons restrains feeding and promotes locomotor activity. Thus, augmenting VTA NtsR1 activity during wakefulness might promote dual behaviors to support weight loss while avoiding any cardiovascular effects. Taken together, these data suggest that augmenting activity of VTA NtsR1 neurons may be useful to support weight loss behaviors without causing psychiatric or cardiovascular liabilities previously described with systemic NtsR1 agonism.

The experimental activation paradigm we used here reveals biological roles for VTA NtsR1 neurons in feeding and locomotor behaviors relevant to control of body weight. However, the potential of this system for treating obesity will depend on whether pharmacological NtsR1 agonists can selectively and safely promote activity of VTA NtsR1 neurons. For example, site-specific agonists to enhance activation of VTA NtsR1 neurons might thwart the increased appetitive drive that occurs after an initial weight loss, preventing regain while encouraging physical activity without adverse cardiorespiratory effects or disrupting mental state. However, this would require targeting NtsR1 agonists selectively to the VTA, so as to avoid inducing off-target modulation of arousal, hypo-locomotor activity, analgesia, body temperature, blood pressure and heart rate associated with Nts-NtsR1 action in other brain

regions[35–38]. There are reports of NtsR1-Dopamine Receptor-1 heterodimers, and if true, it may be possible to design agonists for these heterodimers that might at least confine the drug to the DA system. Whether such heterodimers exist *in vivo*, or how they might signal are vital questions that must be resolved to assess the utility of such an approach. Alternatively, better understanding of NtsR1-mediated signaling may identify strategies to promote beneficial behaviors without adverse physiological effects. Thus, while there is much yet to be learned about the biology of NtsR1 in mediating energy balance, our data support further exploration of VTA NtsR1 neurons as potential pharmacological targets to improve the treatment of obesity and its co-morbidities.

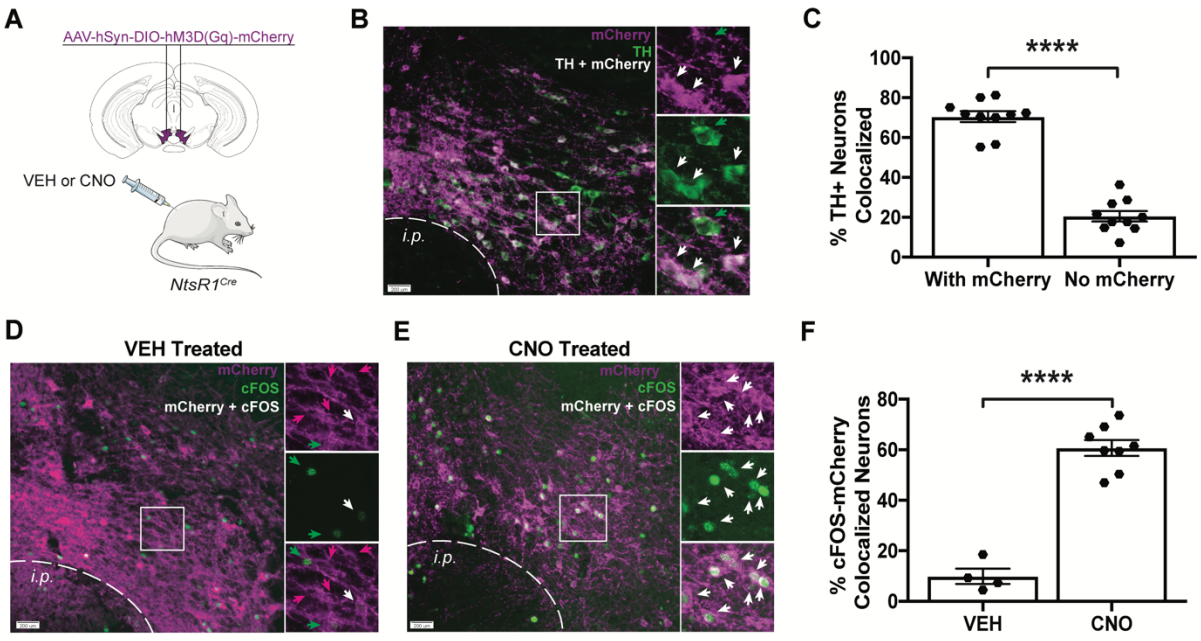

Figure 2.1 VTA NtsR1 Neurons are a Subset of all DA Neurons that can be Activated Using DREADDs. A) *NtsR1*^{Cre} mice were injected in the VTA with AAV2-hSyn-DIO-hM3D(Gq)-mCherry to express hM3Dq-mCherry (excitatory DREADD) in VTA NtsR1 neurons. B) Representative immunofluorescent-labeled images from n = 10 mice showing that tyrosine hydroxylase (TH, marker of DA neurons, green) and mCherry (NtsR1 cells that express hM3Dq-mCherry, purple) are co-expressed in a subset of VTA neurons (white). Panels on right are digital enlargements of boxed region, where white arrows indicate colocalized hM3Dq-mCherry:TH, and green arrows identify TH-only neurons. C) Percentage of TH neurons co-labeled with hM3Dq-mCherry. D-F) Mice were treated with D) VEH (n = 4) or E) CNO (n = 8) 90 minutes before brain collection, followed by immunofluorescent labeling for mCherry (purple) and cFOS (a marker of neuronal depolarization, green). Panels on right are digital enlargements of boxed region, in which magenta arrows indicate neurons that only express NtsR1-hM3Dq-mCherry, green arrows identify cFOS-only neurons and white arrows indicate co-localized mCherry:cFOS neurons.

Figure 2.1 (cont'd)

mCherry-expressing NtsR1 soma are apparent in VEH and CNO-treated brains, but F) CNO significantly increased the percentage of cFOS:mCherry colocalized neurons over VEH treatment. Scale bars = 200 μm. Graphs depict the average percentage of cells ± SEM. ****p < 0.0001 via Student's t-test.

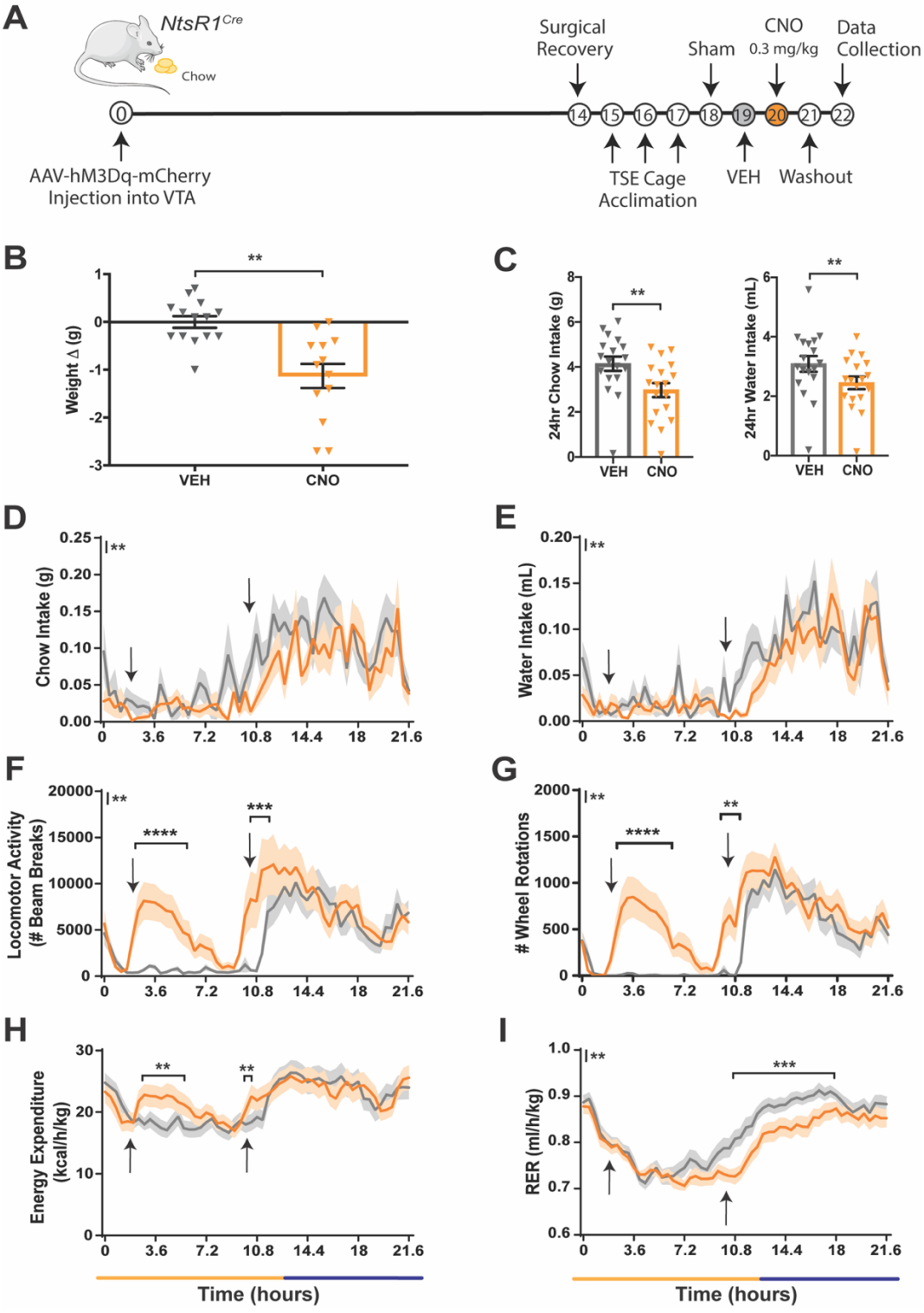

Figure 2.2 Acute Activation of VTA NtsR1 Neurons Promotes Activity and Suppresses Feeding in Normal Weight Mice. Chow fed *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1

Figure 2.2 (cont'd)

neurons were analyzed in metabolic cages while treated with VEH and then CNO. A) Timeline of experiment in days. B) Metabolic cage setup with chow, water and running wheel. C) Change in body weight measured 24 hr after VEH or CNO treatment. For D-I, data is collected every 21-27 minutes and the x axis spans the light (yellow line) and dark cycle (dark blue line) in a ~22-hr period. Arrows identify VEH or CNO injection times. D) Chow intake, E) water intake, F) ambulatory locomotor activity, G) wheel rotations, H) energy expenditure, and I) RER. Graphed data represent mean ± SEM Body weight was analyzed with two-tailed paired t-test (n=13). Metabolic data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests (n=18). Mark next to the y-axis in D-I indicates overall significant overall differences between treatments, see Table 1 for T and F values. P*<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

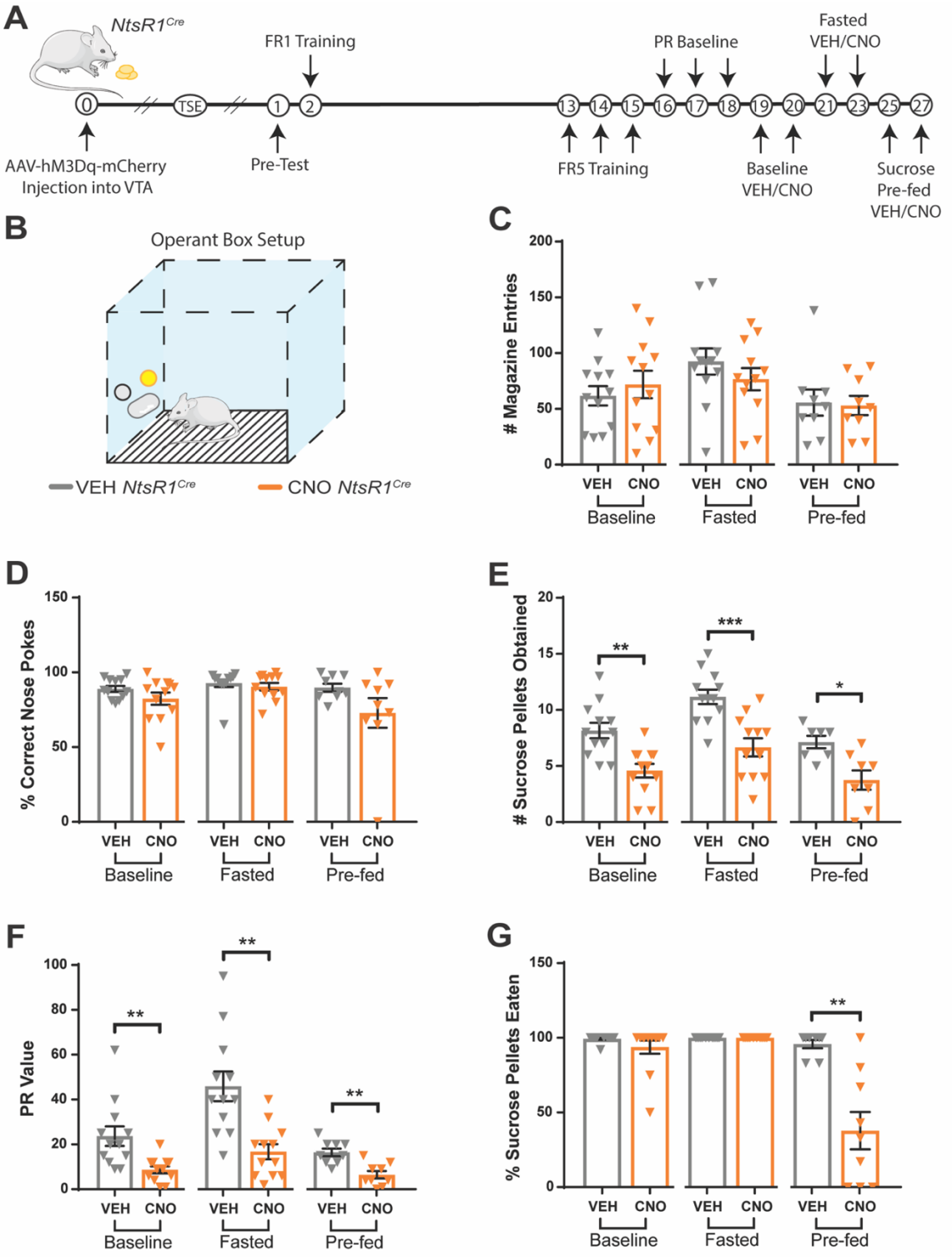

Figure 2.3 VTA NtsR1 Neuron Activation Suppresses Palatable Food Consumption in Normal Weight Mice. A) Timeline of experiment in days. Break in x-axis indicates variable time after

Figure 2.3 (cont'd)

NtsR1^{Cre} mice received intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before beginning the operant test described here. B) Depiction of operant box setup. The position of the correct nose-poke (yellow) was counterbalanced between mice. Chow fed *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1 neurons (n=8-12) were trained to nose poke for sucrose rewards. Mice were then tested on a PR schedule for operant responding after VEH or CNO treatment during baseline (control), fasted (hungry) and sucrose pre-fed (sated) conditions. C) Magazine entries (where sucrose pellet is deposited). D) Correct nose pokes. E) Number of sucrose pellets obtained. F) PR value (last ratio obtained). G) Percentage of sucrose pellets eaten. Data were analyzed by Ordinary One-Way ANOVA with Sidak post-tests, see Table 1 for T and F values. Graphs represent mean ± SEM. P*<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

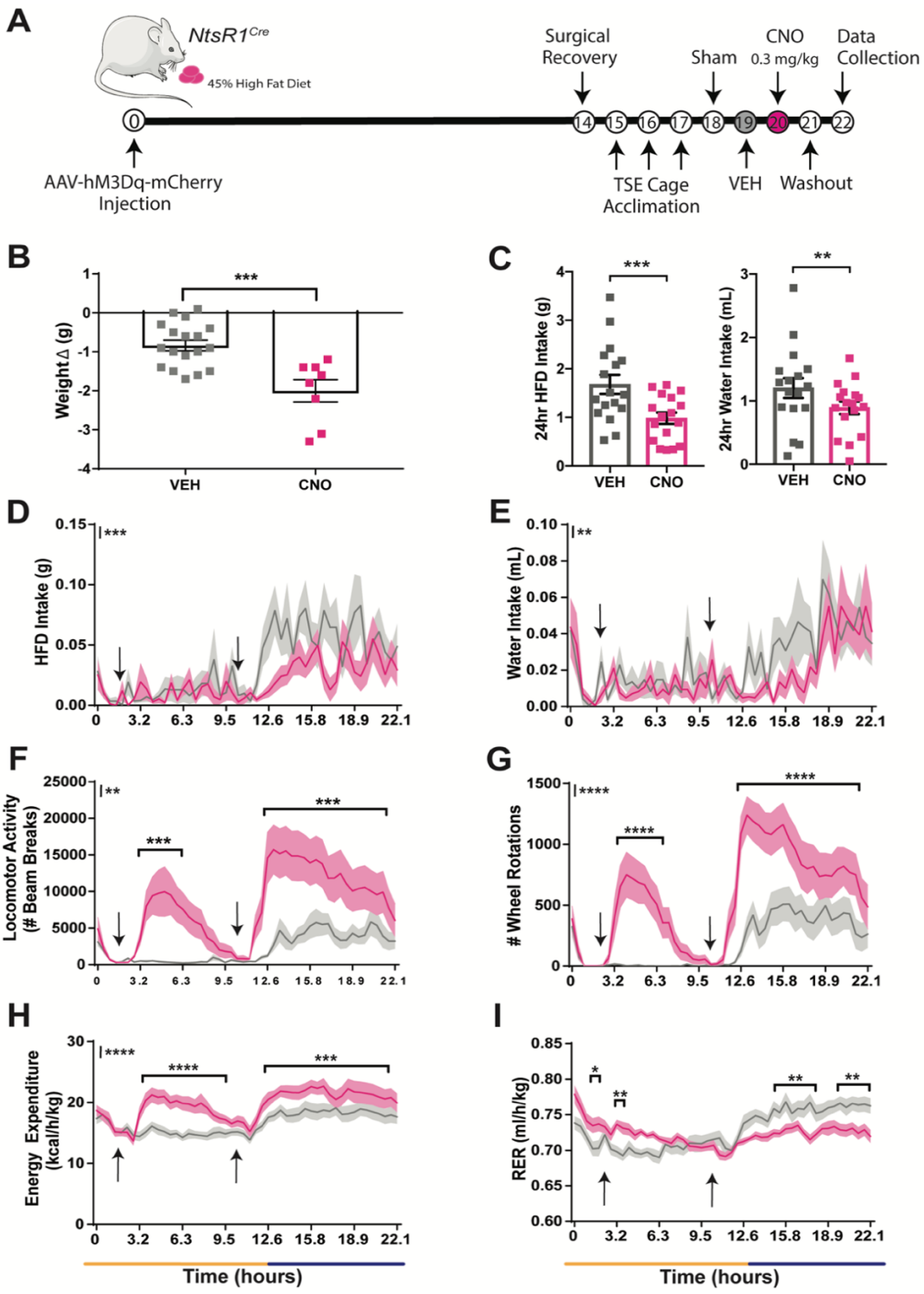

Figure 2.4 Acute Activation of VTA NtsR1 Neurons Promotes Activity and Suppresses Feeding in HFD Mice. VEH or CNO treated HFD *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1

Figure 2.4 (cont'd)

neurons were analyzed in metabolic cages. A) Timeline of experiment in days. B) Metabolic cage setup with 45% HFD, water and running wheel. C) Change in body weight 24 hr after VEH or CNO treatment. In D-I, data is collected every 24-27 minutes and the x axis spans a light (yellow line) and dark cycle (blue line) over a ~22-hr period. Arrows identify VEH or CNO injection times. D) HFD intake. E) Water intake. F) Ambulatory locomotor activity. G) Wheel rotations. H) Energy expenditure. I) RER. Graphed data represent mean ± SEM. Body weight average = 41.7 ± 8.2 g. Body weight was analyzed via two-tailed unpaired t-test (n=8-17). Metabolic data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests (n=17). Mark next to the y-axis in D-I indicates overall significant overall differences between treatments, see Table 1 for T and F values. $P<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

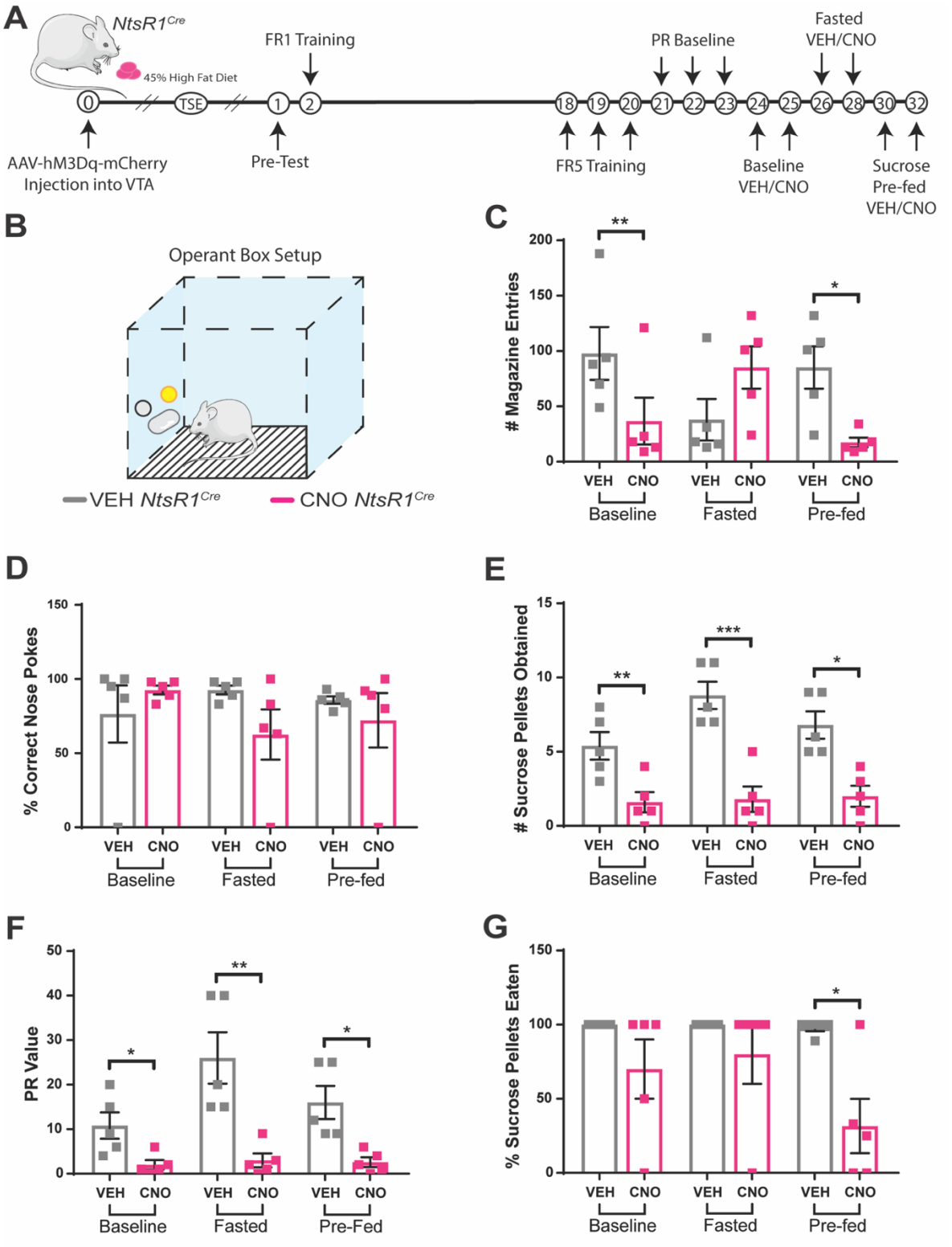

Figure 2.5 Activation of VTA NtsR1 Neurons Suppresses Palatable Food Consumption in HFD Mice. A) Timeline of experiment in days. Break in x-axis indicates variable time after *NtsR1*^{Cre}

Figure 2.5 (cont'd)

mice received intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before beginning the operant testing. B) Depiction of operant box setup. The position of the correct nose-poke (yellow) was counterbalanced between mice. Diet-induced obese *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1 neurons (n=5) were trained to respond for sucrose rewards in operant boxes. Mice were then tested via a PR schedule in response to VEH and CNO treatment during baseline (control), fasted and sucrose pre-fed conditions. C) Magazine entries (where sucrose pellet is deposited). D) Percentage of correct nose pokes. E) Number of sucrose pellets obtained. F) PR value (last ratio obtained). G) Percentage of sucrose pellets eaten. Body weight average = 43.4 ± 10.0 g. Data were analyzed by Ordinary One-Way ANOVA with Sidak post-tests, see Table 1 for T and F values. Graphed data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

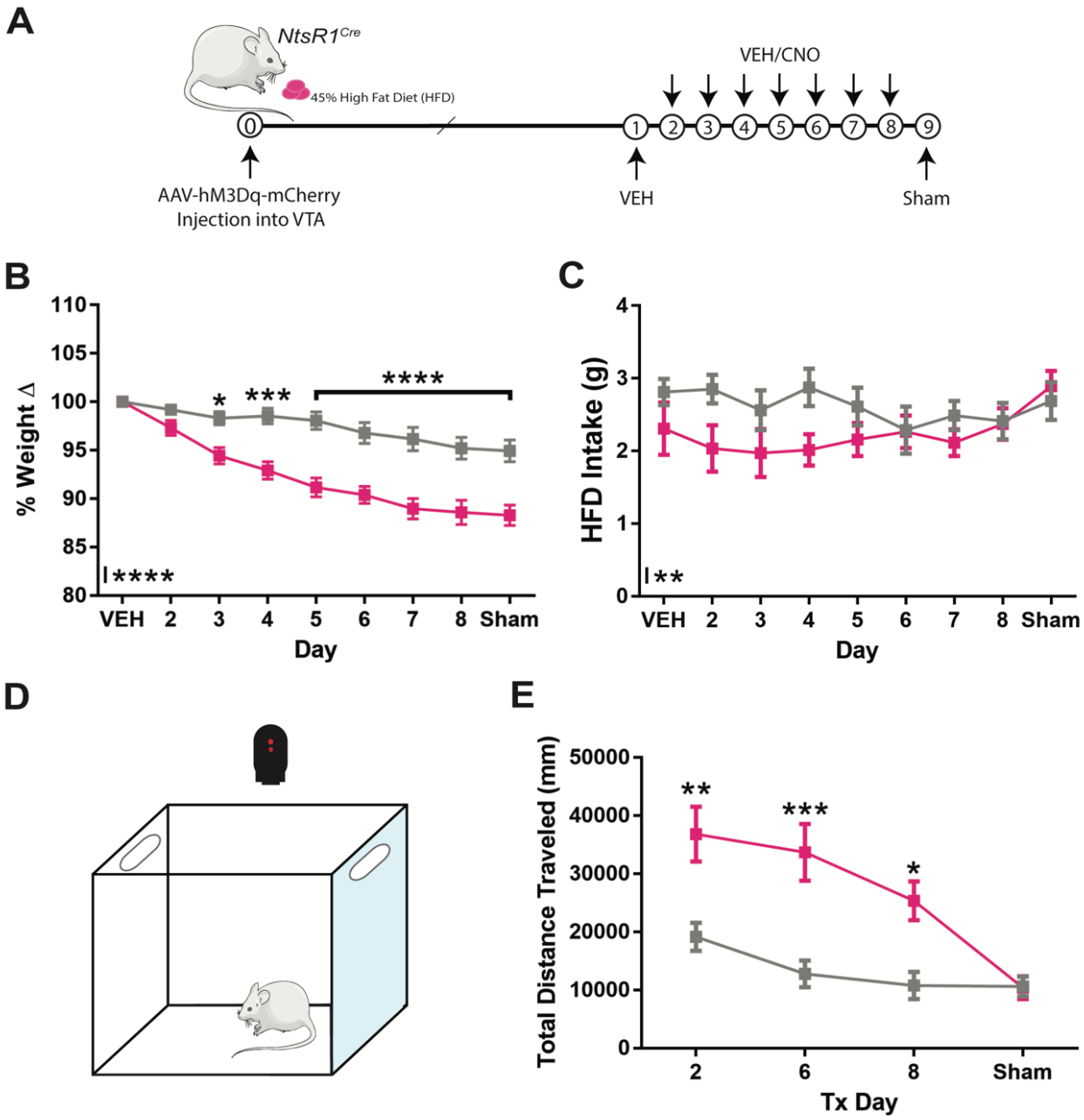

Figure 2.6 VTA NtsR1 Neuronal Activation Promotes Sustained Weight Loss in HFD Mice. Diet-induced obese *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1 neurons (n=9-12) were studied to assess long term (7 days) effects of activating VTA NtsR1 neurons. Mice were treated twice-daily with either VEH or CNO to activate VTA NtsR1 neurons, with *ad libitum* access to 45% HFD. A) Timeline of experiment. Break in x-axis indicates variable time after *NtsR1*^{Cre} mice

Figure 2.6 (cont'd)

received intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before receiving the chronic treatments. B) Percentage weight change over 7 days measured in home cages. C) HFD intake measured in home cages. D) Open field box representation. E) Total distance traveled on days 2, 6, 8 and sham, measured in open field box. Graphed data represent mean ± SEM. Body weight average = 46.3 ± 9.1 g. Data were analyzed by 2-way ANOVA with Sidak post-tests. Mark next to the y-axis indicates overall significant differences between treatments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

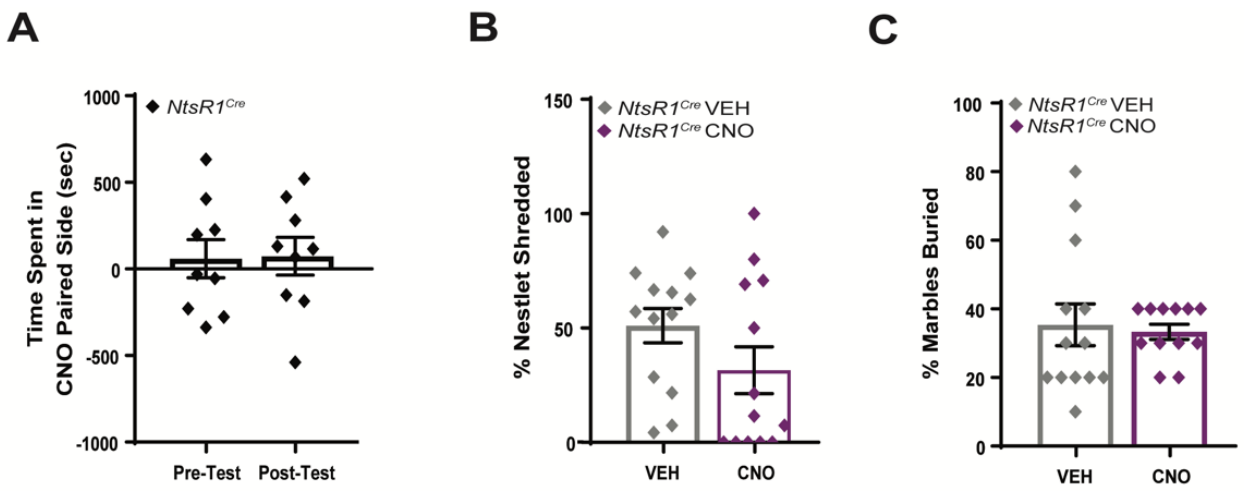

Figure 2.7 VTA NtsR1 Neuronal Activation Does Not Invoke Aversion or Anxiety. A) Normal weight and diet-induced obese *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1 neurons (n=9), and *Wt* littermates (n=4) were assessed via CPP. Mice received CNO in one chamber and VEH treatment in the other, so as to associate the chamber and treatment. Graph depicts time spent in the CNO-paired chamber during the pre- and post-tests, revealing that activation of VTA NtsR1 neurons is neither rewarding nor aversive. Data were analyzed with ordinary 1-way ANOVA with Tukey post-tests. B-D) Anxiety and compulsive like behavior was assessed via nestlet shredding and marble burying. B) Percentage of nestlet shredded; no differences were observed between treatments for *NtsR1*^{Cre} mice (n=13) . C) Percentage of marbles buried; no differences were observed between treatments for *NtsR1*^{Cre} mice (n=13). Data were analyzed with 2-tailed paired t-test. Graphs depict mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

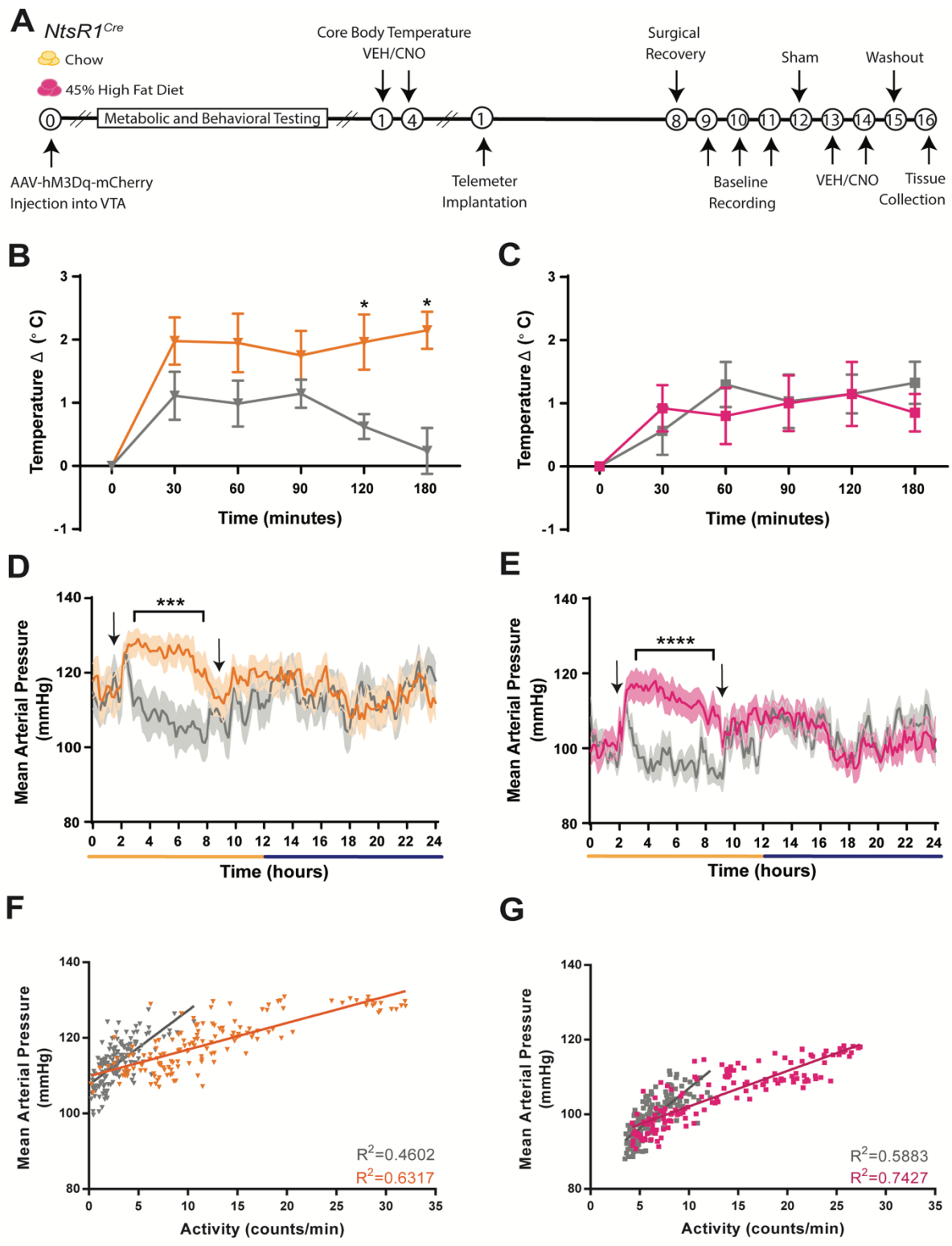

Figure 2.8 Activation of VTA NtsR1 Neurons does not Induce Hypothermia or Vasodepression.

A) Timeline of experiments. Break in x-axis indicates variable time after *NtsR1*^{Cre} mice received

Figure 2.8 (cont'd)

intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before testing. B-C) Core body temperature change every 30 minutes for 120 minutes and at 180 minutes after VEH or CNO treatment in B) normal weight and C) obese mice. D-E) Mean arterial pressure over 24 hr in D) normal weight and E) obese mice. F-G) Activity vs. mean arterial pressure over 24 hr in F) normal weight and G) obese mice. Graphed data represent mean ± SEM. Body weight average for HFD mice = 50.7 ± 11.7 g. Temperature data were analyzed with ordinary 2-way ANOVA and Tukey's posts-tests (chow n=9-10, HFD n=5-6). Telemetry data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests (chow n=11, HFD n=10). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

ACKNOWLEDGEMENTS

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APPENDIX

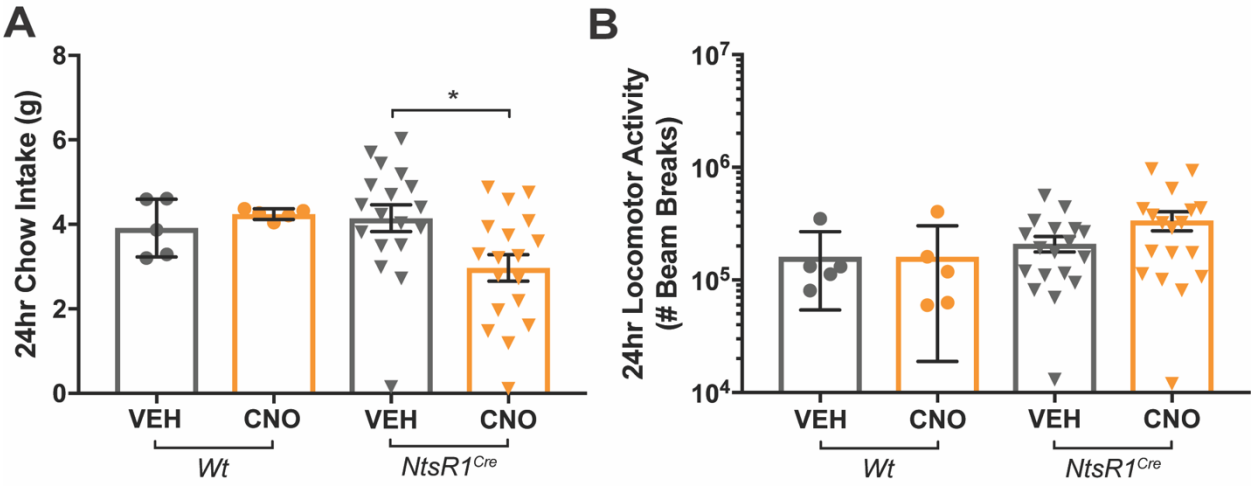

Figure 2.9 Cumulative Food Intake and Ambulatory Locomotor Activity for Chow Fed Mice. VEH or CNO treated chow fed *Wt* (n=5) and *NtsR1*^{Cre} (n=18) mice were analyzed in metabolic cages. In A, 24hr cumulative chow intake. B) 24hr cumulative ambulatory locomotor activity after VEH or CNO treatment. Both A and B were analyzed by Ordinary One-Way ANOVA with Sidak post-tests, and bars represent mean ± SEM.

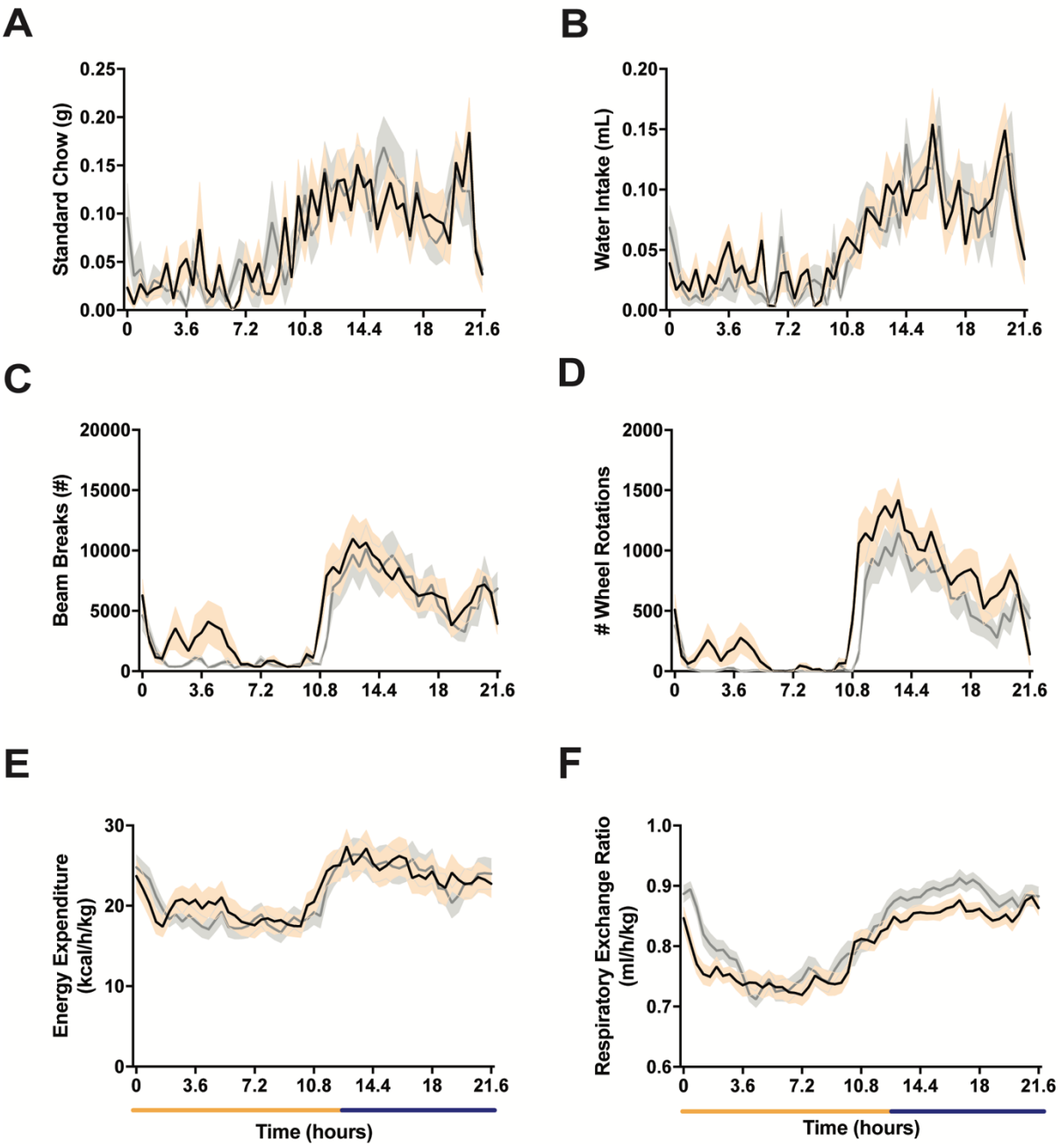

Figure 2.10 Metabolic Behaviors not Persistent After CNO Treatment in Chow-Fed Mice.

Comparison of *NtsR1*^{Cre} mice in metabolic cages in response to VEH treatment vs. no treatment on the washout day (>12 hr after their last CNO injection). Gray lines indicate mice treated with VEH, and black lines indicate un-treated mice (washout day, no injections) after CNO treatment. In A-F, data is collected every 21-27 minutes and the x axis spans both light and dark cycle in a

Figure 2.10 (cont'd)

~22-hr period (n=18). A) Chow intake, B) water intake, C) ambulatory locomotor activity, D) wheel rotations, E) energy expenditure, and F) respiratory exchange ratio. Metabolic data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests, and graphed data represent mean ± SEM.

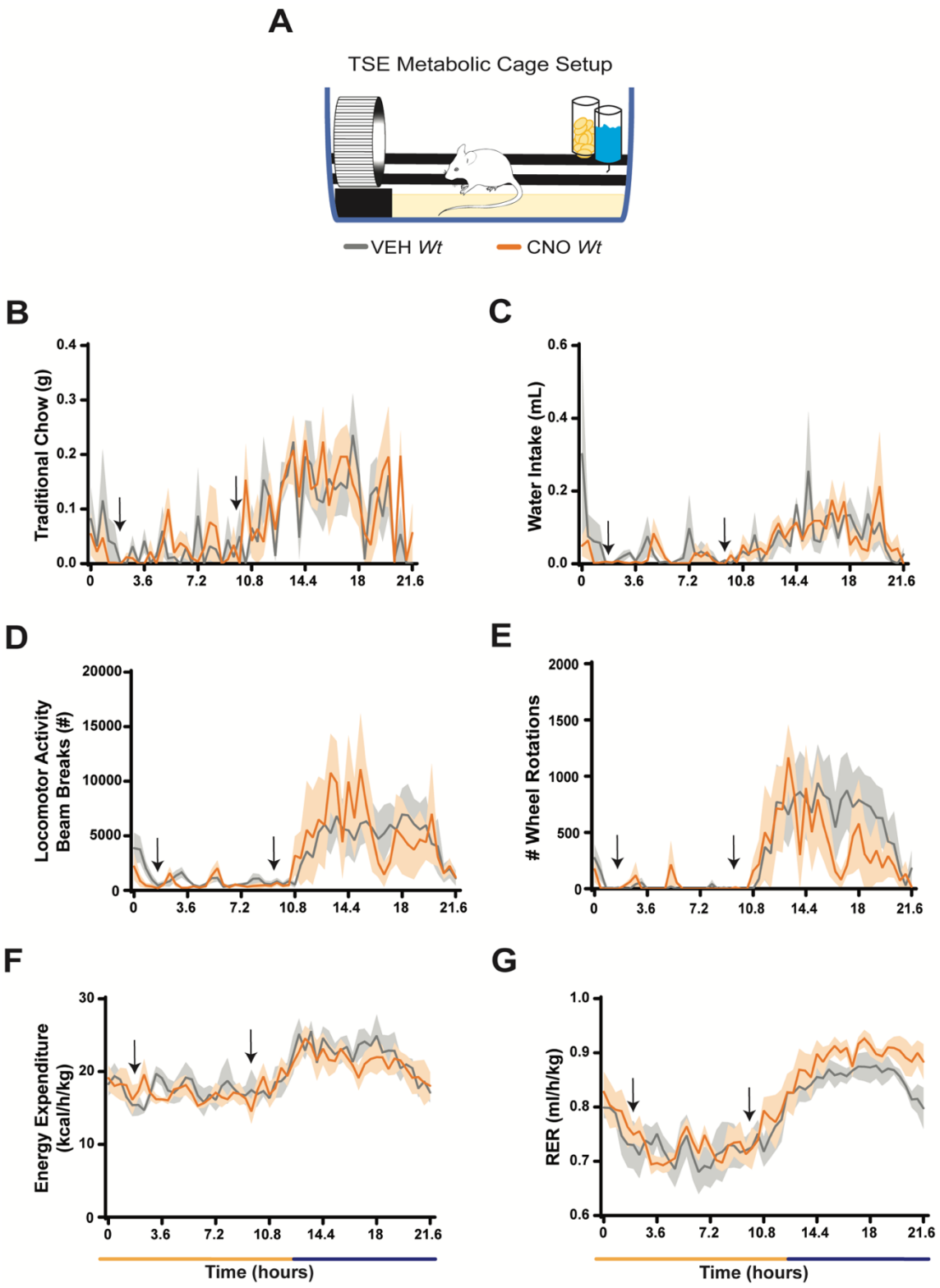

Figure 2.11 CNO Does Not Alter Body Weight or Metabolic Behaviors in WT Chow-Fed Mice.

VEH or CNO treated chow fed *Wt* mice were analyzed in metabolic cages. In A, metabolic cage

Figure 2.11 (cont'd)

setup representation. In B-G, data is collected every 21-27 minutes and the x axis spans both light and dark cycle in a ~22-hr period (n=5). Arrows identify VEH or CNO injection times. B) Chow intake, C) water intake, D) ambulatory locomotor activity, E) wheel rotations, F) energy expenditure, and G) respiratory exchange ratio. Data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests, and graphed data represent mean ± SEM.

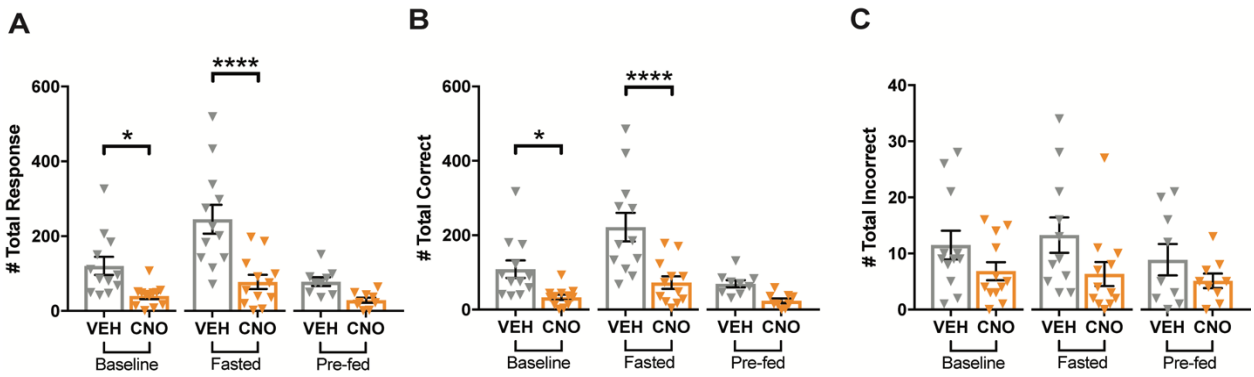

Figure 2.12 Chow Fed Mice Decrease Operant Total Response After CNO treatment. Chow fed *NtsR1*^{Cre} mice (n=7-12) were trained to respond for sucrose rewards in operant boxes, and tested in baseline, fasted and sucrose pre-fed conditions after VEH and CNO treatment. A) Chow mice total response (sum of correct and incorrect nose pokes) for sucrose pellets. B) Chow mice total correct response to sucrose pellets (sum of correct nose pokes). C) Chow mice total incorrect response to sucrose pellets (sum of incorrect nose pokes). Data were analyzed by Ordinary One-Way ANOVA with Sidak post-tests, and bars represent mean ± SEM.

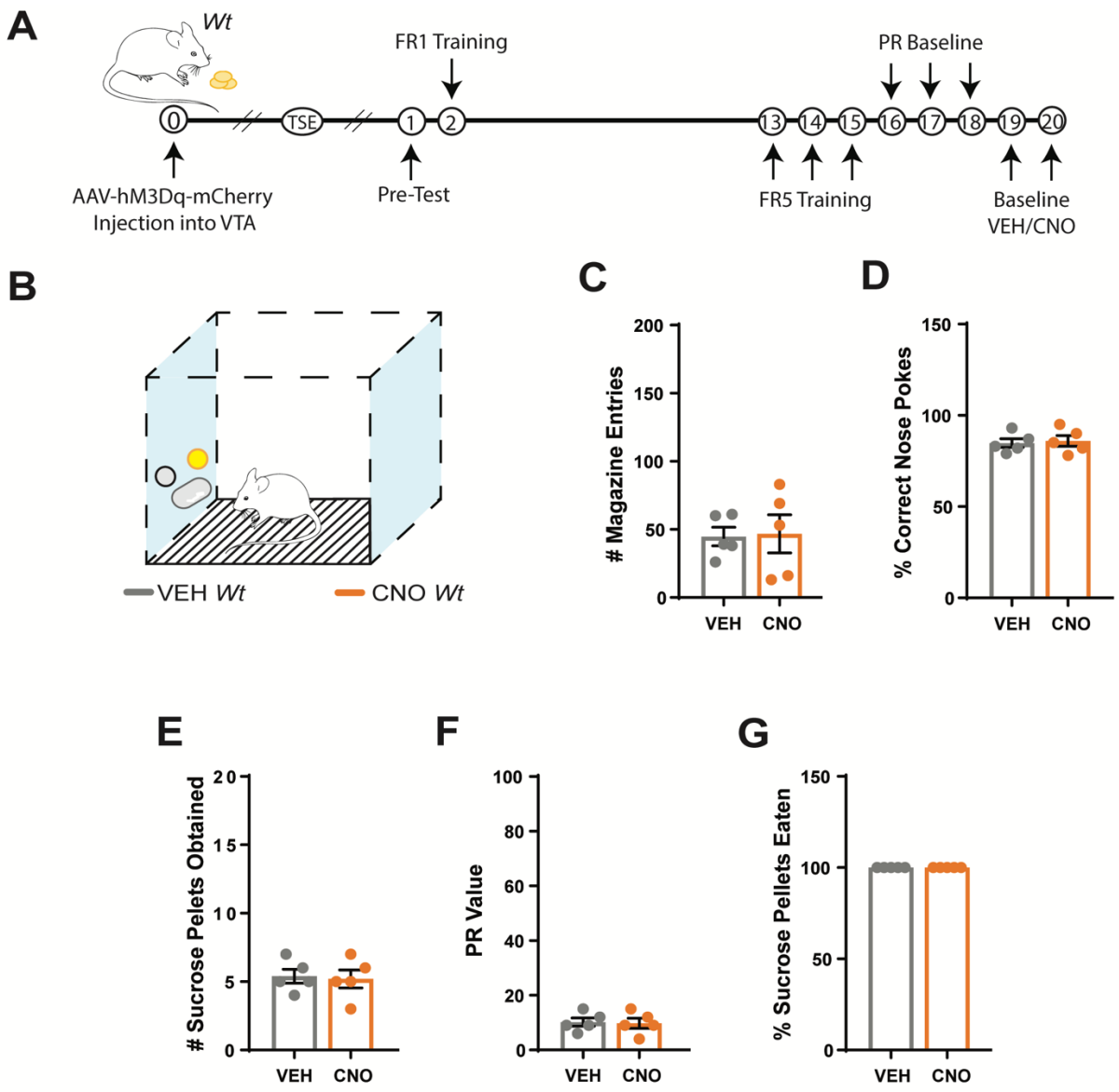

Figure 2.13 Chow Fed *Wt* mice Do Not Respond to CNO to Alter Operant Responding for Rewards. In A, timeline of experiment in days. Break in x-axis indicates variable time after *NtsR1*^{Cre} mice received intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before beginning operant testing. B) Depiction of operant box setup. The position of the correct nose-poke (yellow) was counterbalanced between mice. Chow fed *Wt* mice (n=5) were trained to respond for sucrose rewards in operant boxes, and tested in baseline, fasted and sucrose pre-fed conditions after

Figure 2.13 (cont'd)

VEH and CNO treatment. C) magazine entries, D) correct nose pokes, E) number of sucrose pellets obtained, F) PR value, and G) percentage of sucrose pellets eaten. Data were analyzed with two-tailed paired t-test for each condition, and bars represent mean ± SEM.

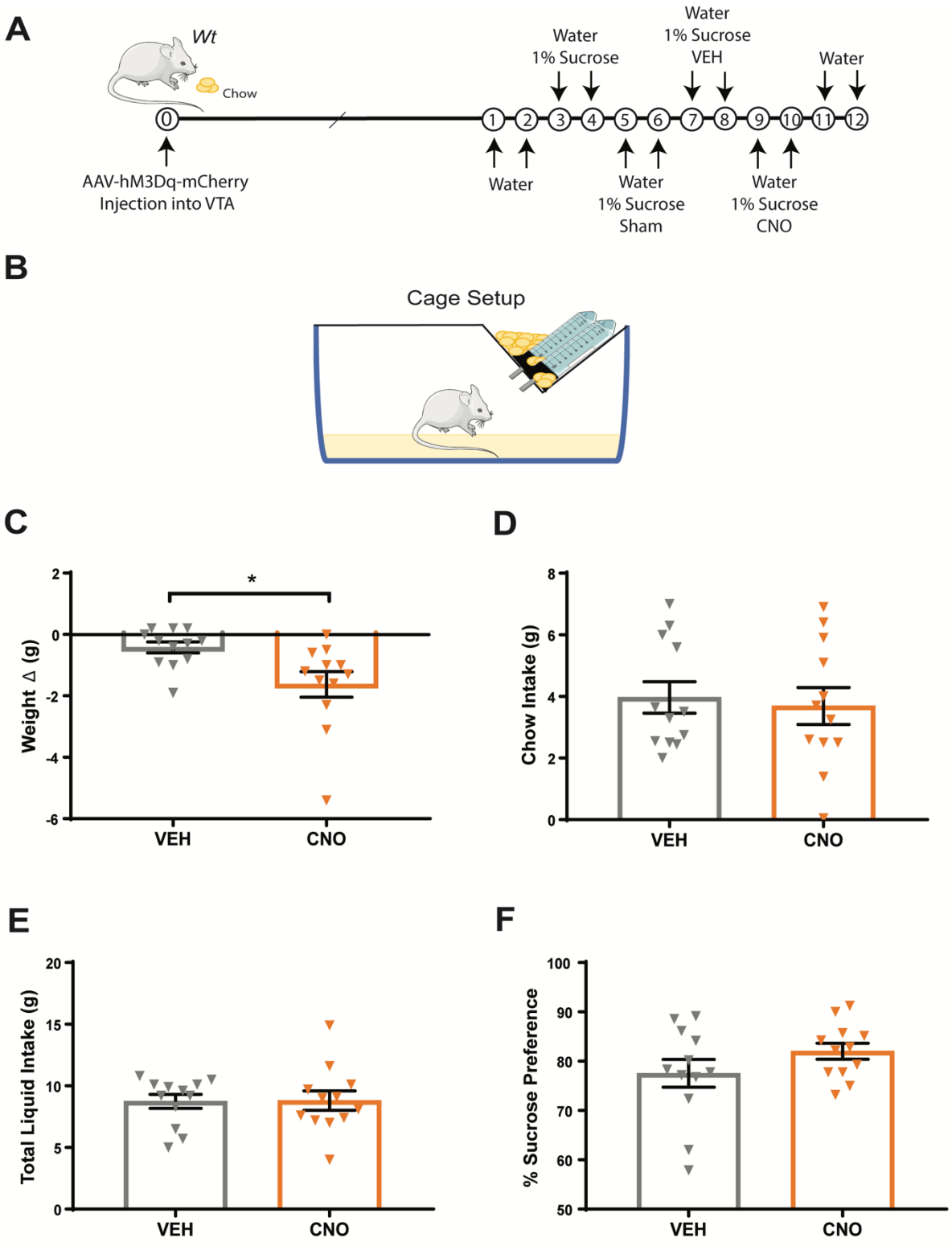

Figure 2.14 VTA NtsR1 Neuronal Activation in Chow Fed Mice does not Alter Sucrose

Preference. Chow fed *NtsR1*^{Cre} mice (n=12) were given the choice for 48hrs between a sipper

Figure 2.14 (cont'd)

bottle containing water and another containing 1% sucrose solution. A) Timeline of experiment. B) Representation of home cage setup with *ad libitum* chow and the 2 bottles. C) Total body weight change, D) chow intake, E) total liquid intake and, F) percentage sucrose preference. Data were analyzed with two-tailed paired Student's t-test and graphed as mean ± SEM. *$p < 0.05$.

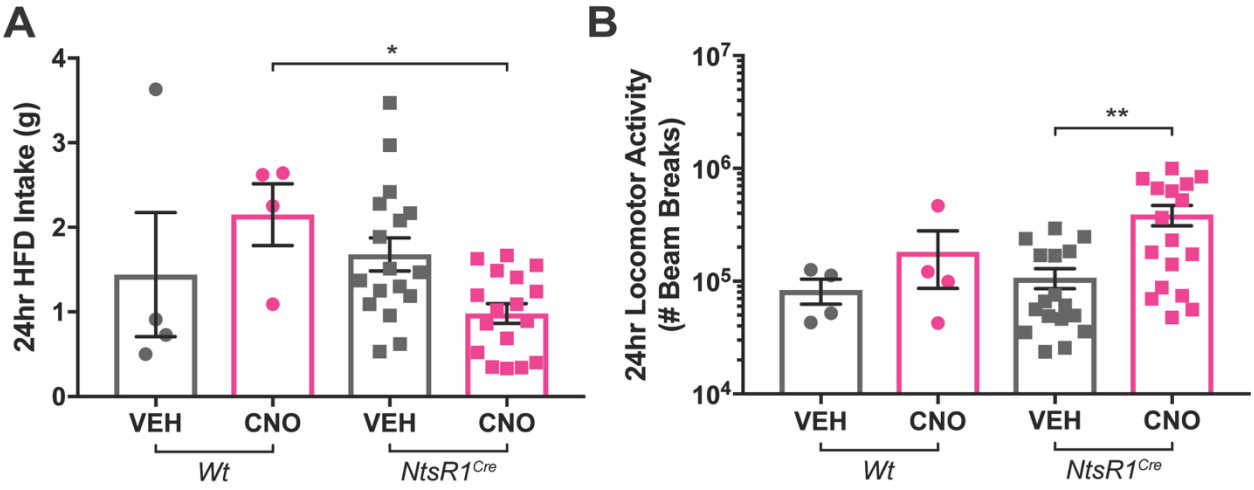

Figure 2.15 Cumulative Food Intake and Ambulatory Locomotor Activity for HFD Mice. VEH or CNO treated chow fed *Wt* (n=4) and *NtsR1*^{Cre} (n=17) mice were analyzed in metabolic cages. A) 24hr cumulative HFD intake. B) 24hr cumulative ambulatory locomotor activity after VEH or CNO treatment. Both A and B were analyzed by Ordinary One-Way ANOVA with Sidak post-tests, and bars represent mean ± SEM.

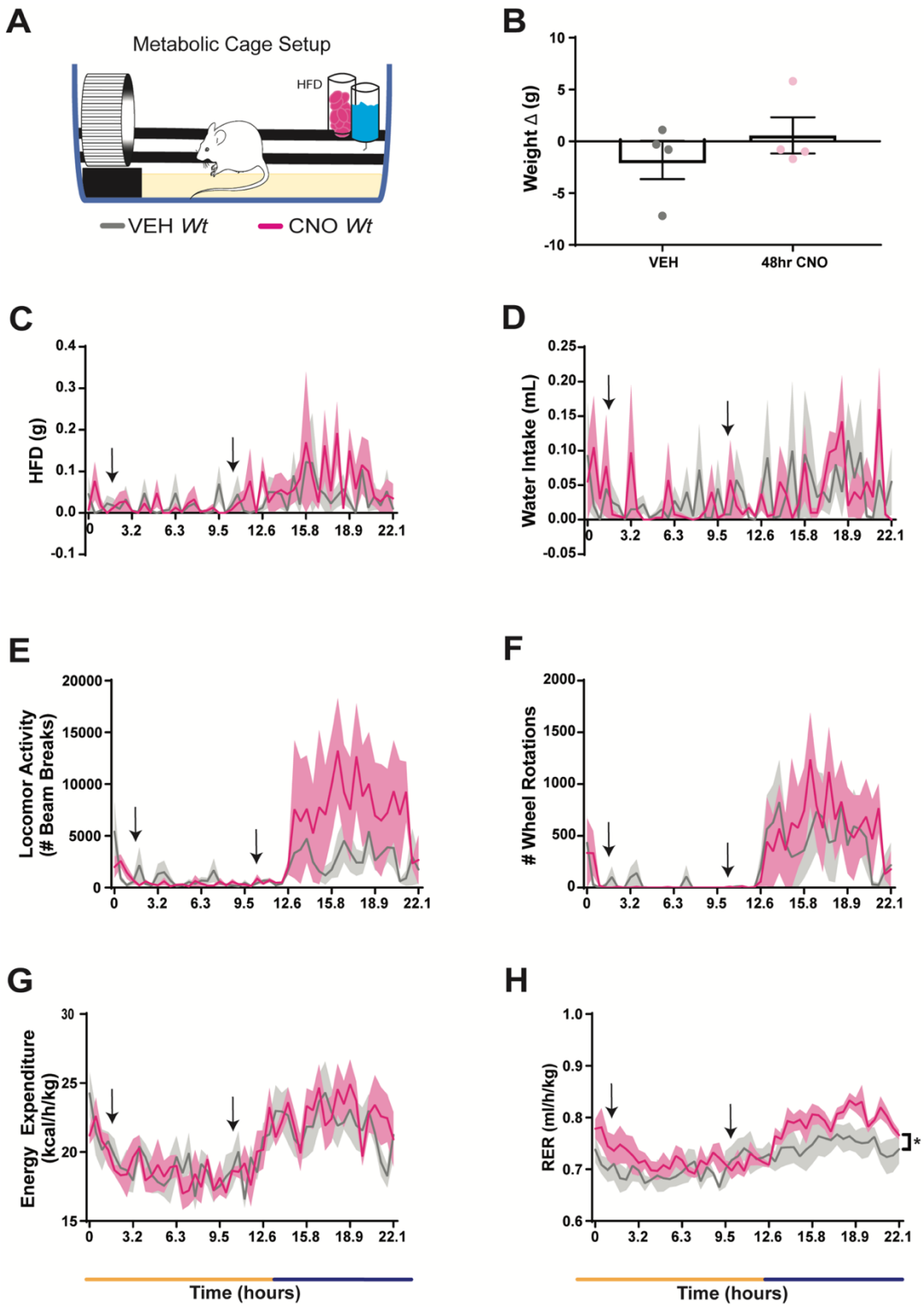

Figure 2.16 CNO Does Not Alter Body Weight in Obese *Wt* Mice. VEH or CNO treated HFD *Wt* mice were analyzed in metabolic cages. In A, metabolic cage setup representation. B) Total

Figure 2.16 (cont'd)

body weight change 24hrs after VEH or CNO treatment (n=4). In C-H, data is collected every 21-27 minutes and the x axis spans both light and dark cycle in a ~22-hr period (n=4). Arrows identify VEH or CNO injection times. C) Chow intake, D) water intake, E) ambulatory locomotor activity, F) wheel rotations, G) energy expenditure, and H) respiratory exchange ratio. Total body weight data was analyzed with two-tailed paired t-test. Metabolic data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests, and graphed data represent mean ± SEM.

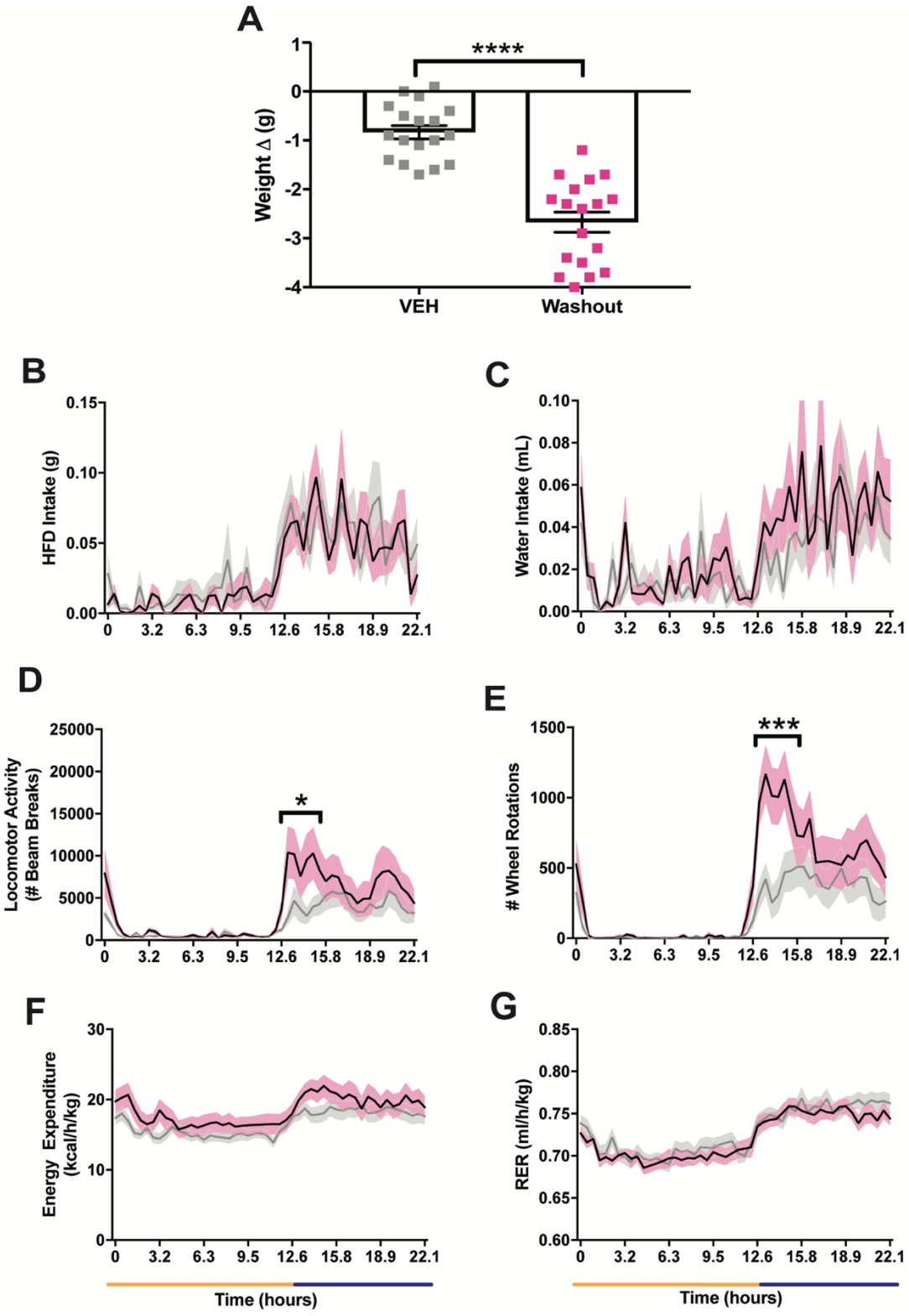

Figure 2.17 Metabolic Behaviors After CNO Treatment in HFD Mice. VEH or not treated HFD *NtsR1*^{Cre} mice were analyzed in metabolic cages. Gray lines indicate mice treated with VEH, and

Figure 2.17 (cont'd)

black lines indicate mice not treated for a day (washout day, no injections) after CNO treatment. In B-G, data is collected every 24-27 minutes and the x axis spans both light and dark cycle in a ~22-hr period (n=17). A) Weight change after VEH treatment and Washout day. B) HFD intake, C) water intake, D) ambulatory locomotor activity, E) wheel rotations, F) energy expenditure, and G) respiratory exchange ratio. Metabolic data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests, and graphed data represent mean ± SEM.

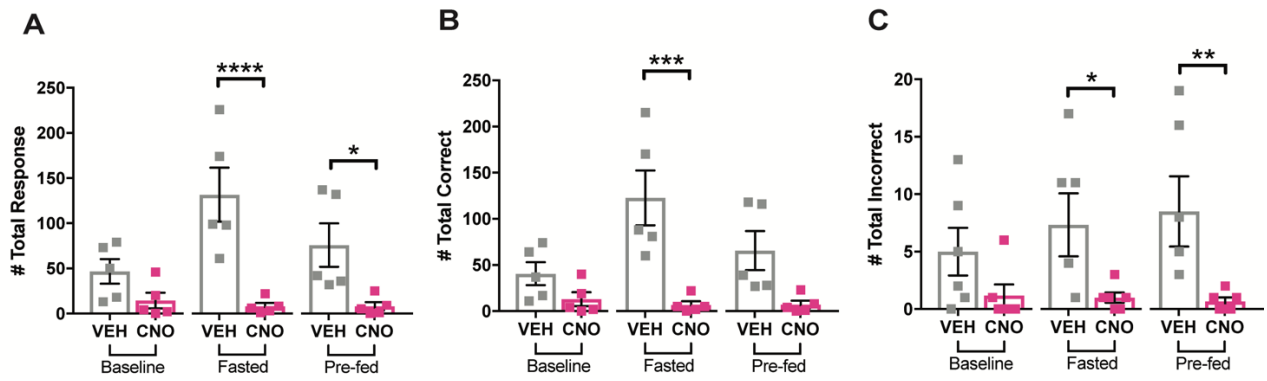

Figure 2.18 HFD Mice Decrease Operant Total Response after CNO treatment. HFD *NtsR1*^{Cre} mice mice (n=5) were trained to respond for sucrose rewards in operant boxes, and tested in baseline, fasted and sucrose pre-fed conditions after VEH and CNO treatment. A) HFD mice total response to sucrose pellets (sum of correct and incorrect nose pokes). B) HFD mice total correct response to sucrose pellets (sum of correct nose pokes). C) HFD mice total incorrect response to sucrose pellets (sum of incorrect nose pokes). Data were analyzed by Ordinary One-Way ANOVA with Sidak post-test, and bars represent mean ± SEM. **p< 0.01.

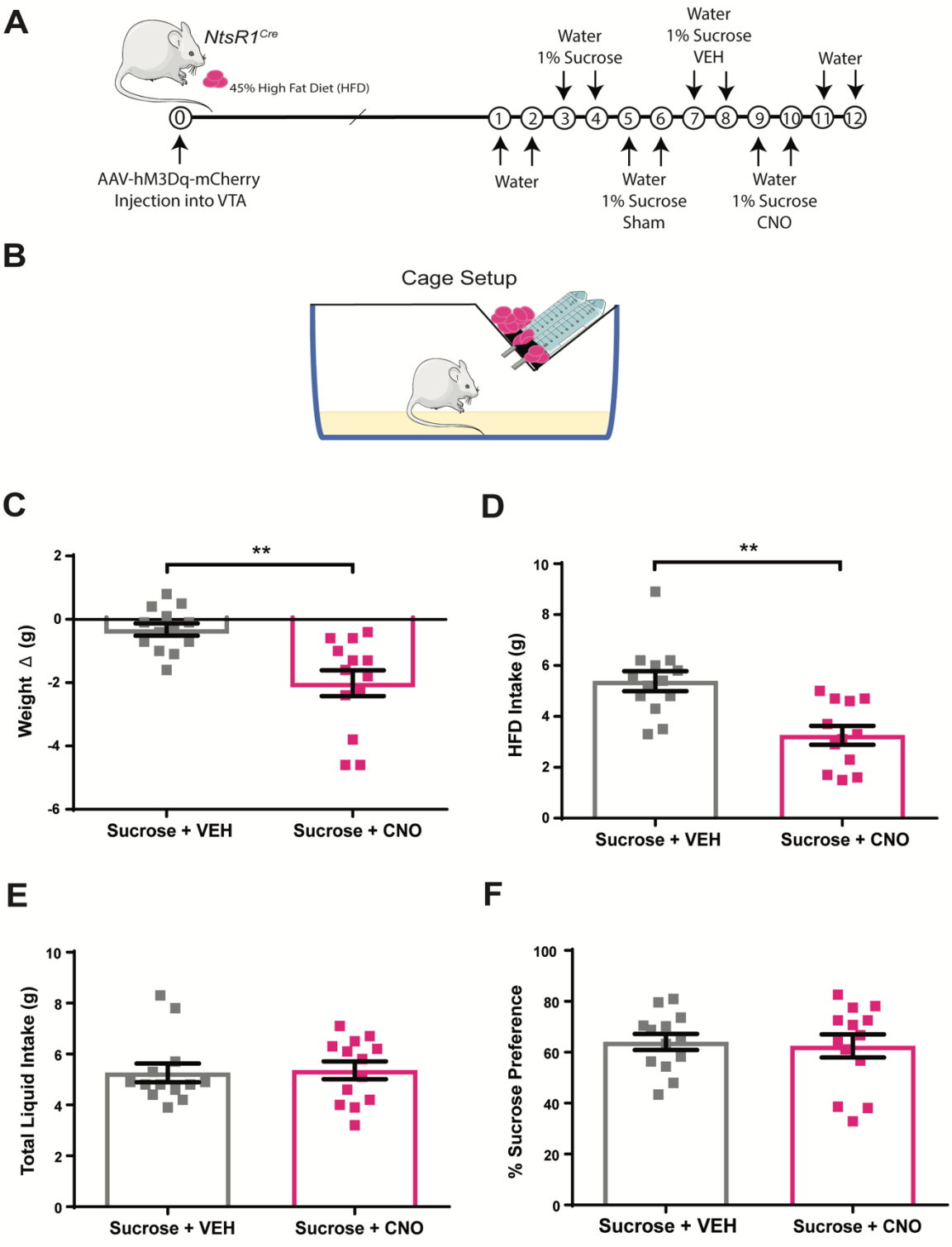

Figure 2.19 VTA NtsR1 Neuronal Activation in Obese Mice does not Alter Sucrose Preference.

Diet-induced obese *NtsR1*^{Cre} mice expressing hM3Dq-mCherry in VTA NtsR1 neurons (n=13)

Figure 2.19 (cont'd)

were given the choice for 48hrs between a sipper bottle containing water and another containing 1% sucrose solution. A) Timeline of experiment. Break in x-axis indicates variable time after *NtsR1*^{Cre} mice received intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before beginning these tests. B) Representation of home cage setup with *ad libitum* HFD and the 2 bottles. C) Total body weight change, D) HFD intake, E) total liquid intake and, F) percentage sucrose preference. Data were analyzed with two-tailed paired t-test and graphed as mean ± SEM.

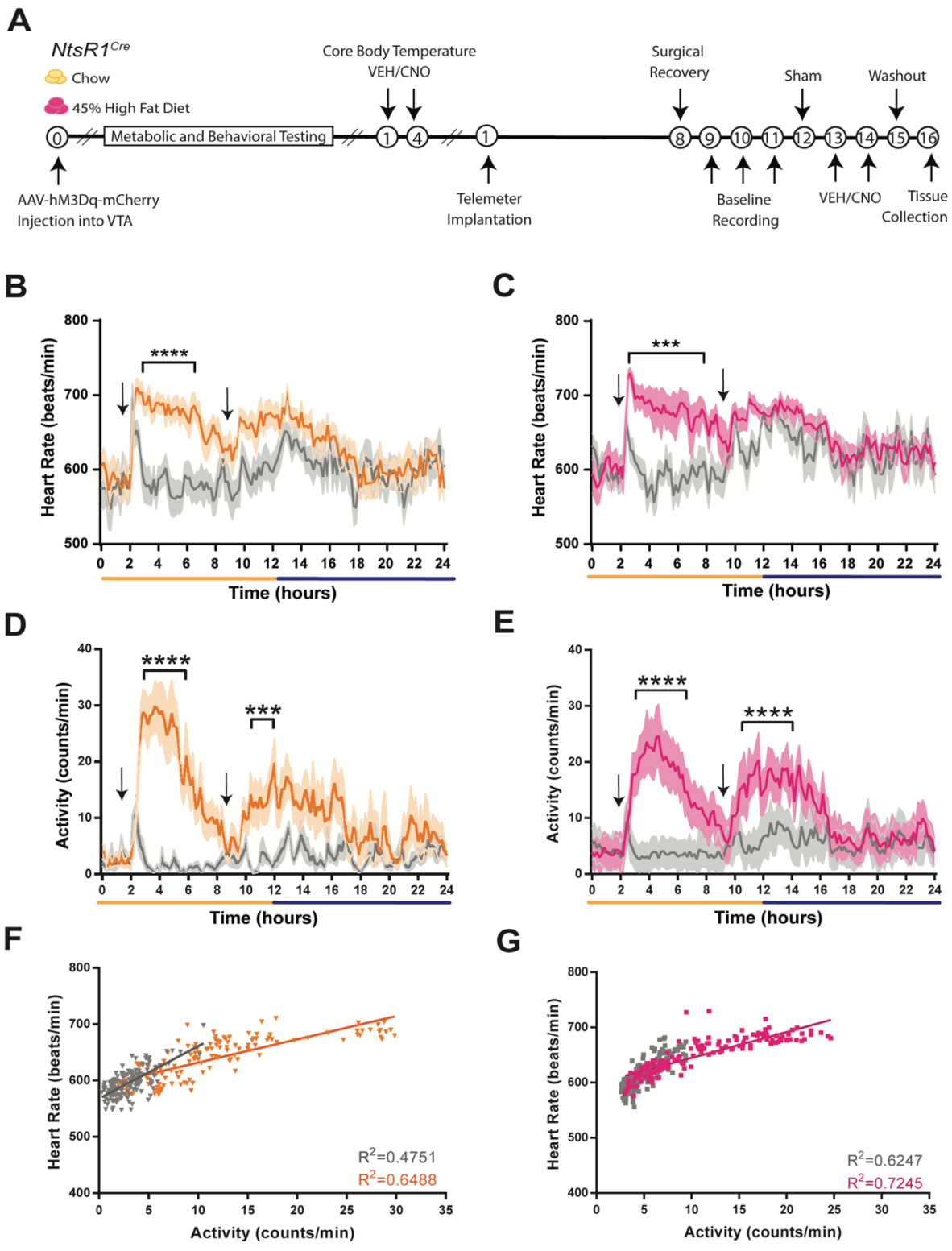

Figure 2.20 Activation of VTA NtsR1 Neurons Elevates Heart Rate as a Function of Activity. A) Timeline of experiment in days. Break in x-axis indicates variable time after *NtsR1*^{Cre} mice

Figure 2.20 (cont'd)

received intra-VTA AAV2-hSyn-DIO-hM3D(Gq)-mCherry before beginning these tests. B-C) Heart rate over 24hr in B) normal weight and C) obese mice *NtsR1*^{Cre} mice. D-E) Activity over 24hr in D) normal weight and E) obese *NtsR1*^{Cre} mice. F-G) Activity vs. mean arterial pressure over 24 hr in F) normal weight and G) obese *NtsR1*^{Cre} mice. Graphed data represent mean ± SEM. Temperature data were analyzed with ordinary 2-way ANOVA and Tukey's posts-tests (normal weight chow-fed mice: n=9-10; obese mice fed HFD: n=5-6). Telemetry data were analyzed by repeated measures 2-way ANOVA with Sidak post-tests (chow n=11, HFD n=10).

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CHAPTER 3. DELETION OF VENTRAL TEGMENTAL AREA NEUROTENSIN RECEPTOR-1 AND ENERGY BALANCE

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ABSTRACT

Central Neurotensin signaling via Neurotensin Receptor-1 (NtsR1) suppresses feeding and promotes locomotor activity - dual weight loss supporting behaviors. However, NtsR1 is widely expressed throughout the brain, and it remains unclear which NtsR1-expressing neurons modulate weight loss vs. other physiology. We previously characterized a subset of ventral tegmental area (VTA) dopamine (DA) neurons that express NtsR1 and activating these neurons in adult mice promotes weight loss. We therefore hypothesized that deleting NtsR1 specifically from VTA DA neurons would promote weight gain by increasing food intake and decreasing physical activity. Curiously, developmental deletion of VTA NtsR1 (by crossing DAT^{Cre} mice with $NtsR1^{flox/flox}$ mice) had no impact on feeding or body weight. Given that NtsR1 may play distinct roles within development vs. adulthood, we then generated mice with adult-onset, conditional deletion of NtsR1 from VTA DA neurons. We injected an adeno associated Cre (AAV-Cre) virus into the VTA of adult $NtsR1^{flox/flox}$ mice to generate mice with site-specific deletion of NtsR1 ($VTAR1^{Null}$ mice) and compared them to mice injected with intact NtsR1 (Control). In contrast to our hypothesis that loss of VTA NtsR1 would increase body weight, $VTAR1^{Null}$ mice had lower body weight than Control mice. Given that VTA DA signaling has also been implicated in the

motivation to obtain food rewards and promoting anxiety and stress we evaluated whether VTAR1^{Null} mice exhibit alterations in food intake, anxiety or physical activity that might reduce their body weight, but found comparable levels to control mice. Altogether, these data suggest that modulating NtsR1 expression in the adult VTA may be useful to safely promote weight loss.

3.1 INTRODUCTION

Energy balance is the coordinated regulation of calories consumed and calories expended. We consume energy through ingesting foods and drinks, and we expend energy through physical exercise and basal metabolism. Energy balance is essential for survival, and the brain organizes the behaviors that are necessary to maintain it. However, although we recognize that the brain coordinates these behaviors, we do not yet understand how. Maintaining energy balance has become more difficult in the increasing prescence of higly palatable, energy dense foods that are easy to consume and lifestyle comforts. These behaviors have contributed to the worldwide rise in overweight and obesity, as recognized by body mass index, and are in fact the most recognized cause for obesity[1]. An increased body mass index elevates the risk of developing severe chronic conditions, such as cardiovascular disease, type-2 diabetes, kidney disease, cancer and disability, and has been accountable for 4 million annual deaths[2,3]. Unfortunately, finding efficacious pharmacotherapies for obese patients has proven particularly difficult because advancements have been limited by the heterogeneity of the disease and an incomplete understanding of how the brain regulates feeding[4,5]. Thus, understanding how the brain coordinates feeding, physical activity and energy expenditure is critical to understand the basic biology of energy balance, and to identify mechanisms to

support sustained weight loss.

Neuropeptides and their receptors have surfaced as important regulators of body weight. In particular, the neuropeptide neurotensin (Nts) is promising because administering Nts centrally suppresses feeding and increases locomotor activity[6–9]. Nts primarily binds to the Neurotensin Receptor-1 (NtsR1), which is a G-protein coupled receptor that is widely expressed thorughout the brain. Nts binding assays in the rodent and human brain suggest that Nts receptors are expressed within the cingulate cortex, midbrain (periaqueductal gray, DR, VTA, SN), subiculum and in the hindbrain (dorsal motor nucleus of the vagus, nucleus of the solitary tract, raphe pallidus, laterodorsal and pedunculopontine tegmental nuclei)[10–12]. NtsR1 is generally coupled to G_q proteins, is predominantly expressed in neurons, and has high affinity for Nts[13,14].

Both Nts and NtsR1 agonists administered centrally restrain feeding and promote dopamine (DA)-dependent locomotor activity[6,15,16]. Moreover, recent work shows that activating site-specific Nts or NtsR1-expressing neurons mediates ingestive behavior without invoking thermoregulatory, pain regulating, and cardiorespiratory effects [15,17–20]. We have characterized a subset of ventral tegmental area (VTA) DA neurons that express NtsR1, henceforth referred to as VTA NtsR1 neurons. VTA NtsR1 neurons may be a pharmacological target for weight loss, as DA neurons in the VTA are essential modulators of feeding and locomotor activity[21,22]. Indeed, activating VTA NtsR1 neurons in normal weight and obese adult mice promotes weight loss by suppressing food intake and increasing locomotor activity[20] (see Chapter 2).

Multiple different strains of NtsR1 knockout mice have been generated, which have produced differing conclusions about the necessity of NtsR1 for regulation of locomotor activity, homeostatic feeding, and body weight. In addition to subtle background and genetic differences between NtsR1 knockout strains, the whole body developmental deletion of NtsR1 in these models may lead to compensatory changes that mask normal action of the receptor[23–26]. This is particularly a concern given that NtsR1 expression varies with age; it is transiently upregulated during gestation and peaks shortly after birth, but is subsequently downregulated as animals reach maturity[27]. In adulthood, NtsR1 levels persist in the VTA, where NtsR1 is expressed primarily in TH+ neurons[20,27–30]. Mice constitutively lacking NtsR1 have been reported to have modest alterations in locomotor activity and chow intake, but have also been shown to overconsume palatable (high fat/high sucrose) food[31]. Together these data suggest that NtsR1 may be more important for restraining consumption of calorie dense foods, with a subtle role in the homeostatic maintenance of food intake.

Given that specific activation of VTA NtsR1 neurons promotes increased locomotor activity and decreased food intake that results in weight loss, we hypothesized that deleting NtsR1 specifically from VTA DA neurons would promote weight gain by increasing food intake and decreasing physical activity. To address this hypothesis, we developed a *NtsR1*$^{flox/flox}$ mouse model and 1) crossed it with DAT^{Cre} mice to delete NtsR1 from all DA expressing neurons from early developmental stages (DATR1^{Null}), or 2) injected it with an adeno associated Cre (AAV-Cre-GFP) virus into the VTA to generate adult-onset, site-specific deletion of NtsR1 (VTAR1^{Null}). Our data suggest that modulating NtsR1 in development and adulthood have different impacts on body weight and may be useful for understanding DA-dependent weight loss behaviors.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 *Mice*

All mice used herein were bred and housed under a 12hr light/12hr dark cycle and were cared for by Campus Animal Resources (CAR). We studied an approximately equal number of male and female mice on the C57/Bl6J background (Jackson Stock #033365). Multiple cohorts of mice were generated, and they were tested via multiple tests, as described below. Cohorts were staggered to control for any seasonal effects. Since we did not observe any metabolic or behavioral differences between males and females in our tests, both sexes were pooled. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health.

Developmental Model: *NtsR1*$^{flox/flox}$ mice were bred to *DAT*^{Cre} mice to generate *DATR1*^{Null} mice. DATR1^{Null} mice lack expression of NtsR1 in all DAT expressing neurons from birth. *NtsR1*$^{flox/flox}$, *DAT*^{Cre}, and *NtsR1*$^{+/+}$ (referred to as wild type, *Wt*) littermates were used as controls.

Adult Model: Heterozygous *NtsR1*$^{flox/+}$ mice were mated to produce homozygous *NtsR1*$^{flox/flox}$ mice and *NtsR1*$^{+/+}$ (referred to as wild type, *Wt*) littermates. *NtsR1*$^{flox/flox}$ and *Wt* mice were then injected at ~8wks of age in the VTA (bilaterally) with either AAV-GFP or AAV-Cre-GFP to generate VTAR1^{Null} mice, Wt^{Cre}, *NtsR1*$^{flox/flox;GFP}$ (VTAR1^{GFP}), and Wt^{GFP} mice. VTAR1^{Null} mice thus have an adult-onset deletion of NtsR1 specifically from VTA DA neurons.

3.2.2 *Gene Expression*

Male and female 12-16wk old *NtsR1*$^{flox/flox}$ and *Wt* littermates 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. 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 were calculated by the 2-ΔΔCt method.

3.2.3 *Diet Tracking and Weekly Weighing*

Developmental Model: At 4 weeks of age, study mice were individually housed with *ad libitum* access to water and chow (Harlan Teklad #7913). Chow weight and body weight were taken once per week for DATR1^{Null}, *NtsR1*$^{flox/flox}$, DAT^{Cre}, and *Wt* littermates for 16wks.

Adult Model: *NtsR1*$^{flox/flox}$ mice and *Wt* littermates that underwent surgery at ~8 weeks (described below) were housed individually after surgery and kept on *ad libitum* chow (Harlan Teklad #7913) for 8 weeks and then were switched to *ad libitum* 45% high-fat diet (HFD, Research Diets D12451) for the duration of experiments, unless otherwise specified. Diet weight and body weight were taken once per week starting 2 weeks after surgery and finishing 8 weeks after HFD was given.

3.2.4 ***Surgery***

NtsR1$^{flox/flox}$ mice and *Wt* littermates (8-12 weeks) were anesthetized (isofluorane/oxygen mixture, 2-4%) and given analgesic (Meloxicam, 5mg/kg), prior to bilateral stereotaxic injection of AAV1.hSyn.H1.eGFP-Cre.WPRE.SV40 (AAV-Cre-GFP, U Penn Vector Core) or AAV-GFP into the VTA (100 nL per side, A/P: –3.2, M/L: ±0.48, D/V: –4.65) as per the mouse brain atlas of Paxinos and Franklin[32]. Mice were allowed to recover for at least 2 weeks prior to metabolic and behavioral testing. Brains were examined via posthoc immunostaining for GFP and TH (see immunostaining method below) to verify targeting and GFP expression in VTA TH+ neurons. *Injected* mice were only included in the final data if GFP-expressing soma were confined to the VTA, and any mice with GFP soma beyond the VTA were excluded.

3.2.5 ***Metabolic Analysis***

Both developmental and adult models were analyzed in PhenoMaster metabolic cages (TSE Systems). Metabolic cages continuously monitored food and water intake, locomotor activity, wheel running, and metabolic parameters (VO2, respiratory exchange ratio [RER], and energy expenditure). Mice acclimated in cages for 48hr prior to testing day. Weight was measured before mice were placed in TSE cages. Ambient temperature was maintained at 20–23°C and airflow rate was adjusted to maintain an oxygen differential around 0.3% at resting conditions. Developmental model mice were tested after 16wks on chow diet. Adult model mice were analyzed at the end of 8wks on chow and then again at the end of 8wks on HFD.

3.2.6 ***Fasting Induced Re-Feeding***

Chow or HFD was removed from home cages ~5PM and mice were given a clean cage bottom. Mice had *ad libitum* access to water during food-deprivation. The following morning ~9AM, fasted mice were given chow or HFD pellets back. Food intake, and body weight was measured 1.5hr, 12hr and 24hr after food was restored. Developmental model mice were tested after 16wks on chow diet. Adult model mice were analyzed at the end of 8wks on chow diet and then again at the end of 8wks on HFD.

3.2.7 ***Operant Testing***

Only developmental model mice were used for this study. Mice were trained to nose-poke for unflavored 20 mg sucrose pellets (TestDiet 1811555) in operant-responding chambers (Med Associates) as previously described[28,33]. *Training*: Mice were food restricted to 85% of their body weight during FR1 training sessions, which occurred over 10-16 consecutive days. Each FR1 training session was terminated after 1hr or when the mouse had earned 50 rewards. Once mice achieved 75% response accuracy with $\geq$20 rewards earned on 3 consecutive days of FR1 training, they were switched to *ad libitum* chow and trained on an FR5 schedule for 3 consecutive days. Mice that failed to reach FR1 criteria after 16 days were removed from the study. *Progressive Ratio (PR) Testing:* After FR5 testing mice were subject to a progressive ratio (PR) schedule where PR=$[5e^{(R*0.2)}]-5$ with R=number of food rewards earned+1. The PR breakpoint was recorded as the highest ratio completed for each 1 hr test session. Mice were

tested until they achieved stable PR, defined as <10% variation in rewards earned over 3 consecutive sessions. To determine if hunger altered responding, mice were fasted overnight, and then tested on the PR schedule.

3.2.8 ***Open Field and Amphetamine Trial***

Developmental and adult HFD model mice were placed in a quiet room under red lights for an hour before placement in open field boxes. For open field, locomotor activity was measured for 30 min using a digital CCD camera and video-tracking software (Clever Sys)[34] but only the last 10-15 minutes was analyzed. For amphetamine (AMPH) trials, developmental model mice were injected with PBS ~8 minutes after being placed in the box and then again with AMPH at the ~40-minute mark. Developmental model mice were tracked for a total of 70 minutes. Adult HFD model mice were treated with PBS immediately before being placed in the boxes, and then with AMPH at the ~30-minute mark. Adult HFD model mice were tracked for a total of 90 minutes.

3.2.9 ***Nestlet Shredding***

Developmental and adult HFD model mice were placed in a quiet room in clean home cage and any nestlet material or enrichment was removed overnight. On testing day (light cycle), food and water were removed, and a pre-weighed, new cotton nestlet was added to the cage. After 30 minutes, intact remnants of the nestlet were removed from the cage and

weighed. If the nestlet was found to be wet, it was air dried overnight and weighed the following day.

3.2.10 ***Marble Burying***

Developmental and adult model mice on HFD were placed in a quiet room in home cage and any nestlet material or enrichment was removed overnight. On testing day (light cycle), mice were placed in the middle of a clean cage with 10 evenly dispersed marbles on top of the bedding. After 30 minutes mice were returned to their home cage. Photos taken before and after the test were used to determine the number and percentage of marbles buried. A marble was counted as buried if 2/3 of the marble was covered.

3.2.11 ***RNAScope***

Adult model mouse brains (n=3) were extracted, postfixed in 10% formalin overnight at 4°C and dehydrated with 30% sucrose in PBS for 2-3 days. Brains were then coronally sectioned (30µm) for RNAScope, as per[30]. Three free floating sections of the VTA per mouse were selected for application of RNAScope single-plex assay (catalog #322360, Advanced Cell Diagnostics) per the manufacturer's protocol. Sections were washed in 1X PBS and incubated in Pretreatment 1 (H_2O_2) at RT until bubbling stops (45-60 min) followed by 0.5X PBS wash and mounting on positively charged slides. Following washing in dH_2O and drying at 60°C oven, sections were incubated in 1X Pretreatment 2 (Target Retrieval Agent) for 5-10 min at 99-104°C and then

washed with dH_2O, dried at RT, dipped in 100% EtOH and air dried. Next, they were incubated in Pretreatment III solution (Protease Plus) for 15 min at 40°C, followed by dH_2O wash. Sections were then incubated in NtsR1 (cat #: 422411, Advanced Cell Diagnostics) probes for 2 h in a humidified oven at 40°C. After amplification steps (Amp1-6), hybridization was visualized by application of Fast-Red-A and Red-B (60:1) for 15-20 minutes. Finally, after washing Fast-Red solution, slides were dehydrated by dipping into xylene and cover-slipped with antifade mounting agent.

3.2.12 ***Perfusion and Immunofluorescence***

Mice received a lethal *i.p.* dose of pentobarbital (Fatal Plus, Vortech) 90 minutes after VEH or CNO treatment, followed by transcardial perfusion with 0.2M PBS (pH 7.4) and then 10% neutral-buffered formalin (Fisher Scientific). Brains were removed, postfixed, and sectioned as described in section 2.2.10. Sections were exposed to primary antibodies including chicken-GFP (1:2000, Abcam, ab13970) and mouse anti-TH (1:1000, Millipore, AB_2201528) followed by species-specific secondary antibodies conjugated to Alexa-568 (1:200, LifeTech, AB_2534017) or Alexa-488 (1:200, Jackson ImmunoResearch, 703-545-155). Sections mounted onto slides were analyzed via an Olympus BX53 fluorescence microscope with FITC and Texas Red filters. Images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera and analyzed using Photoshop (Adobe). Masks applied in Photoshop to enhance brightness and/or contrast were applied uniformly to all samples.

3.2.13 *Statistics*

Unpaired Student's t-tests and ordinary 2-way ANOVA with Sidak post-test were calculated using Prism 7 (GraphPad). A p-value of <0.05 was considered statistically significant. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.3 RESULTS

3.3.1 Developmental Deletion of NtsR1 from DAT-Expressing Neurons does not Alter Body Weight or Chow Intake

To determine how developmental deletion of NtsR1 from DAT expressing neurons impacts body weight, we measured the body weight and food intake of *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice once a week for 16 weeks while in their individual home cages (Figure 3.1 A-B). Deleting NtsR1 from all DAT expressing neurons did not alter body weight over 16 weeks while on chow diet (Figure 3.1 A). Although DATR1^{Null} mice did exhibit a trend toward decreased chow intake compared to *NtsR1*$^{flox/flox}$ mice there were no statistically significant differences between the genotypes (Figure 3.1 B).

3.3.2 Developmental Deletion of NtsR1 from DA Neurons does not Alter Body Composition or Metabolic Phenotype

To determine how developmental deletion of NtsR1 from DAT-expressing neurons impacts energy balance, we analyzed *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice via a body composition analyzer and metabolic chambers (Figure 3.2 A-H). Absolute body weights for *NtsR1*$^{flox/flox}$ and DATR1^{Null} were not significantly different from each other (Figure 3.2 A), but *NtsR1*$^{flox/flox}$ mice have a higher percentage of lean muscle compared to DATR1^{Null} littermates (Figure 3.2 B). Data from metabolic chambers were separated first by dark and light cycles (data not shown separately), but since no genotype differences were found during dark or light cycles, graphed bars represent 24 hrs. Developmental deletion of NtsR1 from DAT-expressing neurons did not alter chow intake, water intake, ambulatory activity, wheel rotations, energy expenditure or RER during 24hr of testing (Figure 3.2 C-H). Although there was a trend for decreased chow intake and wheel rotations in DATR1^{Null} mice compared to NtsR1flox/flox mice, they did not reach the level of statistical significance, chow p=0.09, wheel p=0.12. These data suggest that developmental deletion of NtsR1 from DA neurons is not sufficient to alter metabolic phenotype.

3.3.3 Developmental Deletion of NtsR1 from DA Neurons does not Increase DA-Dependent Food Consumption

NtsR1 has been implicated in suppressing feeding and promoting weight loss, and activation of all LHA Nts neurons suppresses food intake via a NtsR1 dependent mechanism[15]. This includes fasting-induced re-feeding and motivated sucrose responding[15]. Thus, we examined whether developmental deletion of NtsR1 from DA neurons altered the ability of DATR1^{Null} mice to coordinate ingestive behavior and consume palatable food (Figure 3.3 A-H). Developmental deletion of NtsR1 from DA neurons did not alter body weight (Figure 3.3 A) or chow refeeding (Figure 3.3 B) after overnight fasting. When DATR1^{Null} mice were placed in operant chambers (Figure 3.3 C), they did not differ from *NtsR1$^{flox/flox}$* mice in magazine entries or percent of correct nose pokes in baseline and fasted conditions (Figure 3.3 D, E). These data suggest that developmental NtsR1 deletion did not alter anticipatory behavior and that the mice were able to execute the operant task correctly. The number of sucrose pellets mice obtained, the PR breakpoint, and number of sucrose pellets consumed in both the baseline and fasted states were also similar between *NtsR1$^{flox/flox}$* and DATR1^{Null} mice (Figure 3.3 F-H). Overall, these results suggest that developmental loss of NtsR1 in DAT-expressing neurons does not modify the palatability of food or alter the goal-directed behavior needed to obtain it.

3.3.4 Developmental Deletion of NtsR1 from DA neurons Increases DA-Dependent Locomotor Activity Without Inducing Anxiety

We next analyzed mice in open field chambers to assess whether DA-dependent locomotor activity and anxiety behaviors were different after developmental deletion of NtsR1 from DA neurons (Figure 3.4 A-F). DATR1^{Null} mice traveled more distance when placed in the novel open field environment as compared to *NtsR1$^{flox/flox}$* littermates (Figure 3.4 B), which suggests that they have increased exploratory behavior. However, they did not spend more time in the periphery compared to their littermates, which suggests that developmental deletion of NtsR1 does not make mice more or less averse to open spaces compared to *NtsR1$^{flox/flox}$* littermates (Figure 3.4 C). Prior work showed that ablation of VTA NtsR1 neurons modifies amphetamine (AMPH)-induced (DA-mediated) locomotor activity (hyperactivity)[28], thus we examined whether AMPH similar altered locomotor activity in mice with developmental deletion of NtsR1 from DA neurons, so as to assess the integrity of the mesolimbic DA system. We found that open field locomotor activity was not disrupted by PBS-injection stress, but that after AMPH injection (4mg/kg *i.p.*), DATR1^{Null} mice maintained significantly higher locomotor activity/traveled more distance (Figure 3.4 D). These data indicate that loss of NtsR1 from DAT expressing neurons may enhance DA signaling, similar to compensatory increases in extracellular DA that occur with partial loss of DA neurons[35]. Although hyperactivity and excessive DA release have been associated with anxiety, we found no differences in anxiety-like behaviors between DATR1^{Null} and *NtsR1$^{flox/flox}$* mice as assessed via

peripheral/central time (Figure 3.4 C), nestlet shredding and elevated plus maze (EPM) (Figure 3.4 E, F).

3.3.5 Introducing Flox Increases VTA NtsR1 Gene Expression, but AAV-Cre-GFP is Effective at Deleting VTA NtsR1.

We then examined whether introducing flox sequences around exon 1 of the NtsR1 gene could impact the integrity of VTA DA neurons. Thus, we analyzed the fold difference in gene expression between *Wt* and *NtsR1*$^{flox/flox}$ mice for DA-associated transcripts (Figure 3.5 A-C). TH (Figure 3.5 A) and DAT (Figure 3.5 B) were not significantly different between genotypes. NtsR1, however, was significantly increased in the VTA of *NtsR1*$^{flox/flox}$ mice (Figure 3.5 C). These data suggest that introducing flox sequences around NtsR1 neurons increases NtsR1 expression but does not modify expression of key components of the VTA DA system (TH, DAT).

To verify that we could site-selectively delete NtsR1 in adult mice, we then administered unilateral VTA injections of either AAV-GFP or AAV-Cre-GFP to adult *Wt* and *NtsR1*$^{flox/flox}$ mice and analyzed for NtsR1 mRNA expression by RNAScope assay (Figure 3.5 E, D). Depiction of AAV-Cre injection into VTA of adult Wt and *NtsR1*$^{flox/flox}$ mice (Figure 3.5 E). NtsR1 mRNA (white) was detected in the VTA of AAV-GFP and AAV-Cre-GFP injected *Wt* mice (Figure 3.5 D-A) but not in AAV-Cre-GFP injected adult *NtsR1*$^{flox/flox}$ mice (Figure 3.5 D-B). Consistent with this view, *NtsR1*$^{flox/flox}$ mice did not show expression of NtsR1 mRNA in the VTA side injected with AAV-Cre-GFP.

3.3.6 VTA Specific Deletion of NtsR1 from Adult Mice Decreases Body Weight but not Food Intake

To determine how deletion of NtsR1 from established VTA DA neurons impacts body weight, we measured the body weight and food intake of VTAR1^{GFP} and VTAR1^{Null} mice over 8wks while in their individual home cages (Figure 3.6). Adult-onset deletion of NtsR1 from VTA neurons resulted in a modest reduction of overall lower body weight of VTAR1^{Null} mice while on chow and HFD as compared to VTAR1^{GFP} controls (Figure 3.6 A, B; y axis asterisks indicate overall treatment effect). By contrast, no differences in body weight were observed in *Wt* mice injected with AAV-Cre-GFP or AAV-GFP (data not shown). However, VTA DA deletion of NtsR1 did not reduce overall cumulative chow or HFD intake (Figure 3.6 E, F). These data suggest that NtsR1 is important for behaviors that influence body weight independent of regular food intake.

3.3.7 Deletion of NtsR1 from Adult DA Neurons Decreases DA-Dependent Food Intake and Weight Gain

Food restriction decreases baseline DA levels in efferent target sites and enhances dopamine release in response to food rewards. Since NtsR1 expressing neurons are a subpopulation of all VTA DA neurons, we assessed whether deleting NtsR1 from VTA DA neurons changed DA-mediated food intake. VTAR1^{GFP} and VTAR1^{Null} mice were fasted overnight and then re-fed the next day while on chow and HFD (Figure 3.7 A-D). The change in body

weight of VTAR1^{Null} mice did not significantly differ from VTAR1^{GFP} mice after chow refeeding, but VTAR1^{Null} mice did consume significantly less chow overall, particularly 24hr after fasting (Figure 3.7 A, B). While on HFD however, both overall body weight change and HFD intake were significantly lower for VTAR1^{Null} mice when compared to VTAR1^{GFP} mice.

3.3.8 NtsR1 Deletion From Adult VTA DA Neurons does not Alter DA-Dependent Locomotor Activity or Anxiety Behaviors

We next used open field chambers to assess whether DA-dependent locomotor activity and anxiety behaviors were different after targeted adult-onset deletion of NtsR1 from DA neurons (Figure 3.8 A-F). When place in the novel open field environment, VTAR1^{Null} mice and VTAR1^{GFP} mice traveled comparable total distance and spent a similar percentage of time in the periphery (Figure 3.8 A, B). This suggests that VTAR1^{Null} mice do not have increased exploratory behavior or higher locomotor activity. We then used amphetamine (AMPH)-induced locomotor activity to assess the integrity of the mesolimbic DA system in VTAR1^{Null} mice. We found that open field locomotor activity was not disrupted by PBS-injection stress, and AMPH injection (4mg/kg i.p.) elicited comparable increases in locomotor activity in both VTAR1^{Null} and VTAR1^{GFP} mice compared to PBS injections (Figure 3.8 C). Although hyperactivity and excessive DA have been linked with anxiety, we found no differences in anxiety-like behaviors between VTAR1^{Null} and VTAR1^{GFP} mice as assessed via nestlet shredding, elevated plus maze (EPM) and marble burying (Figure 3.8 D-F).

3.4 DISCUSSION

Studies with germline, whole body NtsR1 knockout mice suggest that there is a role for NtsR1 in regulating energy balance behaviors. However, the requirement for NtsR1 in these behaviors has not been clear, largely due to different NtsR1 knockout strains and compensatory changes that may mask normal NtsR1 action. NtsR1 may also regulate distinct aspects of developmental and adult physiology, since NtsR1 is transiently upregulated during gestation and peaks shortly after birth, but is subsequently downregulated in most brain areas as animals reach maturity[27]. Notably, essentially all DA neurons within the VTA express NtsR1 transiently during development, but only ~70% of DA neurons retain high levels of NtsR1 in adulthood[20,27,28]. Here we used a new $NtsR1^{flox}$ mouse to explore the requirement for NtsR1 in DA neurons and whether it may differ throughout lifespan. We show that developmentally deleting NtsR1 from all DA expressing neurons (by breeding to a DAT^{Cre} strain) does not promote or impede weight loss behaviors. It does, however, promote some DA-dependent responses, such as sustained increased responding to AMPH and increased exploratory locomotor activity. We also show that deleting NtsR1 in adulthood (using a site-directed AAV-Cre administered into the VTA) promotes lower body weight and reduces motivated food intake in mice fed with both chow and HFD. Moreover, both developmental and adult conditional NtsR1 deletion did not promote anxiety-like behaviors. Our findings hint that germline deletion of NtsR1 causes compensation that does not reflect the requirement of NtsR1 within the adult brain. In contrast, loss of NtsR1 from established DA neurons reveals a modest contribution of NtsR1 to body weight and some DA-dependent behaviors and drive, but

that NtsR1 is not required for homeostatic maintenance of energy balance.

Prior studies have utilized multiple, independently generated whole body NtsR1 knockouts to assess the role of this receptor in energy balance. Depending on the strain and study, these models have yielded equivocal results, from increased or unaltered body weight[24,25,31], increased or decreased food intake[24,25,31], increased, decreased or unaltered locomotor activity[24–26,31,36], and increased AMPH induced activity[26] compared to *Wt* controls on the same background. This heterogeneity of responses may be due in part to differences in the generation of the varied NtsR1 knockout strains, differing genetic backgrounds and different testing regimens. We also reasoned that constitutive deletion of NtsR1 throughout the many cells that transiently express NtsR1 throughout the body might lead to compensations that mask physiology that is specifically regulated by NtsR1-expressing neurons that co-express DA. We therefore hypothesized that targeting NtsR1 deletion to DA neurons and the VTA, which are known to modulate food intake, locomotor activity and body weight might be more useful to define the role of NtsR1 in energy balance. Thus, we generated new *NtsR1$^{flox/flox}$* mice and used them to make two conditional deletion models to study the requirement of NtsR1 in weight loss and weight loss behaviors. We used a developmental model, in which mice lacked germline expression of NtsR1 from all DA-expressing neurons, and an adult model, in which NtsR1 was deleted specifically from the VTA of adult mice. These models enabled us to examine the temporal and site-specific requirement of NtsR1 in DA neurons.

A caveat of this study is that the *NtsR1$^{flox/flox}$* mice necessary for conditional deletion may not be physiologically identical to *Wt* mice. Indeed, this is a concern of any genetically modified

mouse line, as introducing genetic alterations into genomic DNA can influence expression of adjacent genes and cell function[37]. Despite this, it has not yet become common practice to assess gene expression in Cre or flox lines, and it is likely that many lines may in fact differ from *Wt* mice in ways that have yet to be appreciated. Here we investigated the potential impact of introducing loxP sites flanking exon 1 of *Ntsr1,* and found that this significantly increased NtsR1 gene expression compared to *Wt* littermates. Increased expression of NtsR1 could conceivably lead to differences in baseline behavior between *Wt* and *NtsR1*$^{flox/flox}$ mice, although we did not observe significant differences between these genotypes in our study (data not shown). However, to control for this possibility we only compared behaviors of deleted mice with *NtsR1*$^{flox/flox}$ mice, and not with *Wt* mice. Importantly, we did not observe any alterations in TH or DAT in VTA tissue from *NtsR1*$^{flox/flox}$ mice, which are co-expressed by NtsR1-DA neurons, suggesting that the genetic manipulation did not cause generalized disruption of the neurons. Hence, *NtsR1*$^{flox/flox}$ mice are a model to assess the role of NtsR1 in DA-expressing neurons, and the first to enable conditional and temporally-specific deletion of NtsR1 from neurons. Beyond our work here on the role of NtsR1 in DA neurons, this line be a valuable resource to dissect the role of NtsR1 in different cell types throughout the body.

We found that developmental NtsR1 deletion from DA neurons had no effect on energy balance, as it did not alter food intake, locomotor activity, or body weight. Developmental NtsR1 deletion did significantly decrease % lean mass compared to *NtsR1*$^{flox/flox}$ mice, but this difference in body composition did not influence total body weight. Developmental NtsR1 deletion also did not alter operant responding for sucrose (a DA-dependent behavior) nor did it

induce anxiety as assessed by open field, nestlet shredding and EPM. Interestingly, lack of NtsR1 in DA neurons prolonged increased AMPH induced activity, consistent with previous reports on whole body NtsR1 knockout mice[26] but in contrast to reports on mice with ablated VTA NtsR1 neurons[28]. Moreover, developmental model mice exhibit increased locomotor activity in a novel environment, which may be due to DA-mediated arousal. These results suggest that germline deletion of NtsR1 from DA neurons may lead to enhanced DA signaling, similar to compensatory increases in extracellular DA that occur with partial loss of DA neurons[35]. Numerous mechanisms could enhance DA action, such as impaired DAT kinetics or altered balance of D1/D2 that is thought to underlie the hyperdopaminergic phenotype of constitutive NtsR1 knockout mice. Altogether, these data hint that developmental NtsR1 expression in all DA neurons may be more important for establishing circuitry modulating DA-mediated arousal/attention behaviors (e.g., novel object and AMPH responding) but not appetitive behavior. Indeed, DA is important for attention although it is speculated that DA arising from the substantia nigra may be more related to attention processes while DA from the VTA is more important for reward processing[38]. It is important to note that crossing *NtsR1*$^{flox/flox}$ mice to *DAT*^{Cre} leads to cre-mediated deletion germline deletion of NtsR1 from all DA neurons, including those of the VTA and substantia nigra, which may explain the bias of attentional/arousal effects in this line. Although we did not observe different effect in males and females (data is pooled from box sexes), DA signaling can differ in males and females[39], and it is possible that hormonal or environmental context could influence the role of NtsR1-DA neurons, and this should also be explored in the future.

Chemogenetic-mediated activation of VTA NtsR1 neurons largely recapitulates the effects of Nts treatment in the VTA to promote lower body weight[20], hence, we hypothesized that deleting NtsR1 in the VTA would lead to increased food intake and body weight. In contrast to our hypothesis, deleting NtsR1 from the VTA of adult mice caused reduced body weight compared to controls, which was independent of food intake for mice both on chow and HFD. Likewise, the selective ablation of VTA NtsR1 neurons in adult mice protected them from age-related weight gain and diet-induced obesity[28]. However, mice with ablated VTA NtsR1 neurons differed in that they exhibited hyper-locomotor activity and metabolism, and in fact overconsumed food in an attempt to counteract their excessive energy expenditure. By contrast, our current work deleting just the NtsR1 from intact VTA DA neurons results in a modest weight reduction without hyper-activity or over-consumption. It stands to reason that deleting a single GPCR would produce a more modest phenotype compared to killing an entire population of neurons. Yet, the modest impairments in body weight and fasting-induced refeeding observed after adult-onset NtsR1 deletion in the VTA suggests that NtsR1 plays a role in tuning appropriate energy intake behavior and body weight.

Accumulating evidence suggests that the Nts-NtsR1 system does not influence homeostatic feeding, but instead, primarily modulate motivational or DA-dependent feeding. Indeed, we did not observe any impact of deleting NtsR1 from adult VTA DA neurons on normal, *ad libitum* intake of chow. However, since food deprivation increases DA neuron excitability, decreases DA re-uptake, and potentiates DA release during re-feeding[40–45], we tested whether fasting exposed any alterations in DA-mediated refeeding in VTAR1^{Null} mice.

Indeed, VTAR1^{Null} mice on both chow and HFD showed restrained re-feeding, but only HFD had decreased weight gain over a 24hr period. These data suggest that deletion of VTA NtsR1 can suppress chow and HFD feeding and subsequent weight gain in HFD mice even under conditions that increase the drive to feed, such as fasting-induced weight loss. Moreover, these data support the idea that NtsR1 in established VTA neurons primarily impacts DA-mediated, motivated feeding behavior, which would not be at play during *ad libitum* feeding. In this light, the lack of alterations in normal feeding of VTAR1^{Null} mice is understandable. Modulating NtsR1 expression or signaling may conceivably be useful to tune DA-dependent feeding without disrupting homeostasis, which could make it a safer approach for modifying behavior. However, since modulating VTA DA signaling has been implicated in weight loss by promoting anxiety, aversion or reinforcement[46,47], we also evaluated whether deleting VTA NtsR1 causes such adverse DA-associated physiology. Notably, deleting VTA NtsR1 from HFD mice in adulthood did not invoke these conditions. This is also consistent with activation of VTA NtsR1 neurons, which did not invoke anxiety or aversion behaviors[20]. Taken together, these data support that modulating NtsR1 expression or neuronal activity can beneficially promote weight loss without undesirable psychomotor impact.

A key remaining question is why deleting NtsR1 from adult VTA neurons modestly promoted weight loss, while activating such neurons invoked more robust weight loss. This seeming dichotomy may reflect that both methods could promote loss of NtsR1 expression. Activating neurons can cause internalization of GPCRs so it is possible that chemogenetic activation of VTA NtsR1 neurons in fact led to diminished NtsR1 expression, as well as invoking

other signaling pathways that promoted DA release, and both effects may have contributed to weight reduction. In the current study, deleting NtsR1 from adult VTA NtsR1 neurons would only mimic the reduction of NtsR1 without increasing activation of the VTA neurons, and so may reveal only the contributions of losing GPCR signaling via NtsR1. Going forward it will be vital to test how agonist binding to NtsR1 signaling impacts signaling in VTA DA neurons, and whether it biases for sustained intracellular signaling or NtsR1 internalization. Answering these questions is important to understand the mechanism by which NtsR1 contributes to energy balance, and how its signaling might be modulate to bias for weight loss.

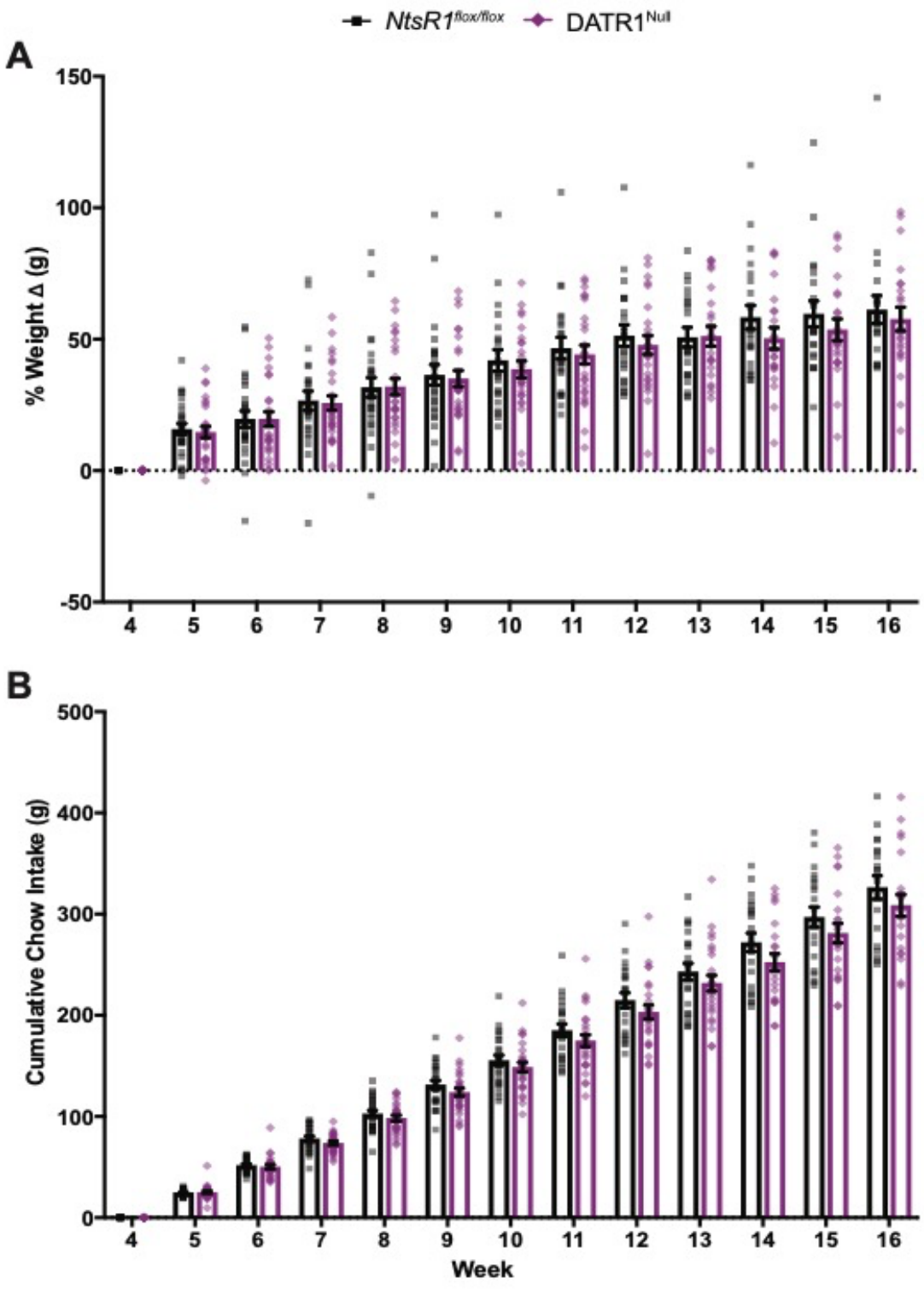

Figure 3.1 Developmental Deletion of NtsR1 does not Influence Body Weight or Food Intake.

Chow fed *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice were individually housed at 4 weeks old and analyzed

Figure 3.1 (cont'd)

in home cages. Body and food weight was taken weekly for 13 weeks. A) % Weight change from week 4 to 16 and B) cumulative chow intake over 13 weeks. Both *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice gain weight and consume chow indistinctly from each other. Data represent mean ± SEM analyzed via ordinary 2Way-ANOVA with Sidak's correction. n=12-13. *p<0.05.

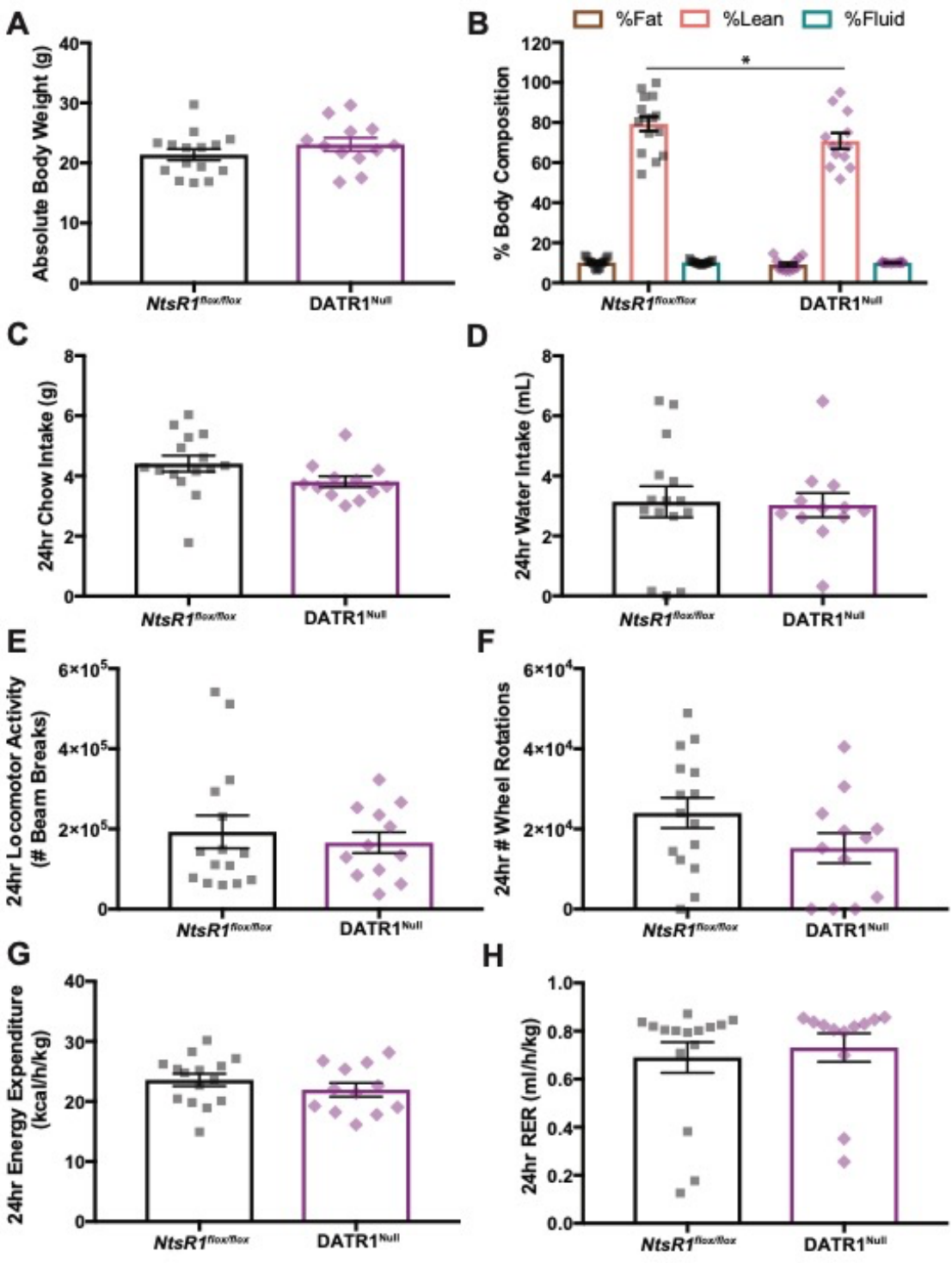

Figure 3.2 Developmental NtsR1 Deletion Alters Body Composition but not Metabolic Phenotype. Chow fed *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice were analyzed in metabolic cages. A) Body weight measured on the day mice were placed in metabolic cages. B) % Body composition

Figure 3.2 (cont'd)

of fat, lean muscle, and fluid. *NtsR1*$^{flox/flox}$ mice have significantly higher lean muscle percentage compared to DATR1^{Null} mice. For C-H, data is collected and pooled for a ~24 hr period that contains both light and dark cycle. Deleting NtsR1 from DAT expressing neurons did not alter C) chow intake, D) water intake, E) ambulatory locomotor activity, F) wheel rotations, G) energy expenditure, and H) RER. Data represent mean ± SEM Body weight was analyzed with two-tailed unpaired t-test (n=12-13). *$p<0.05$.

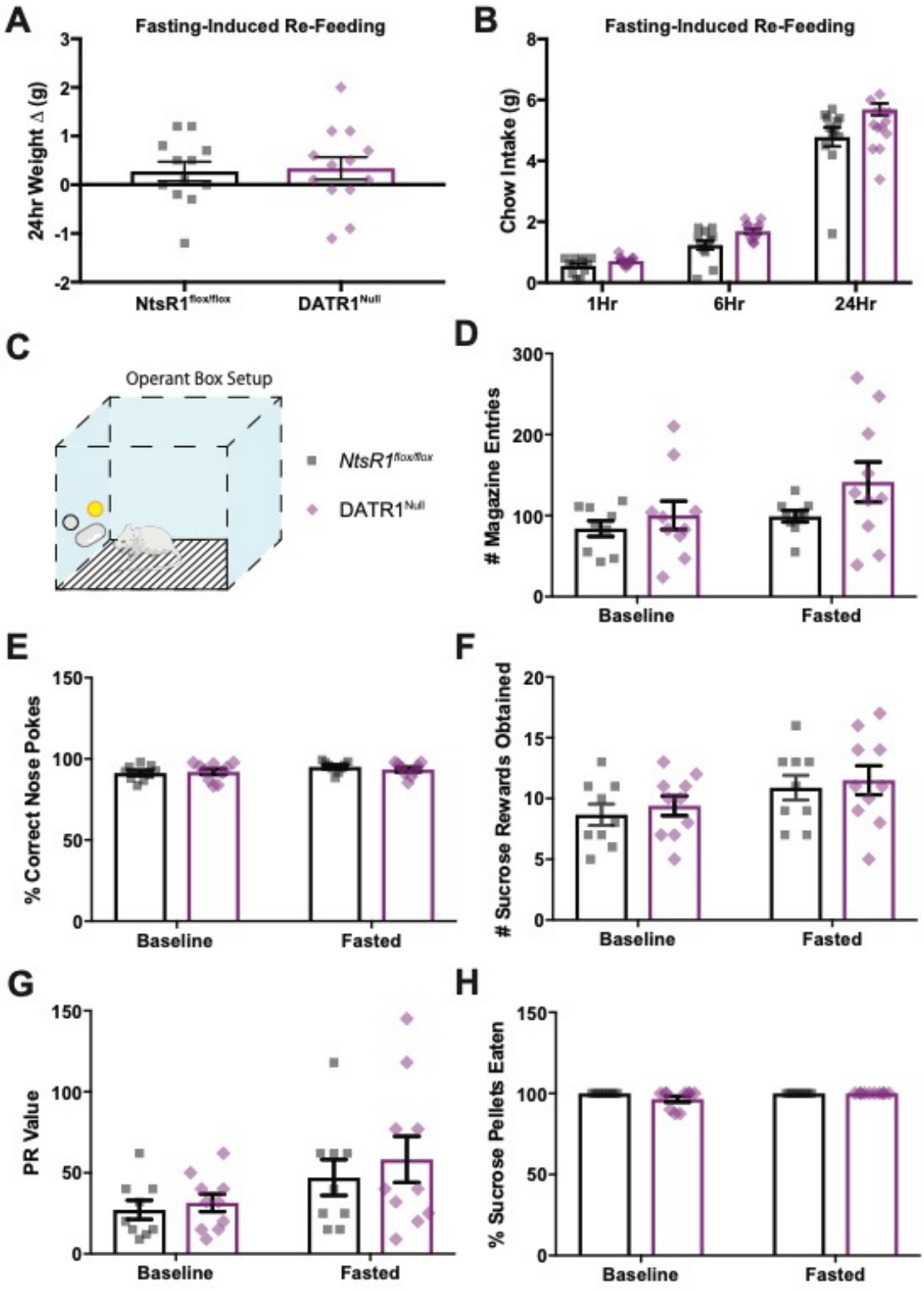

Figure 3.3 Deleting NtsR1 from DAT Expressing Neurons does not Increase DA-Dependent Food Consumption. *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice were fasted overnight, then re-fed the next

Figure 3.3 (cont'd)

day with normal chow diet and then tested in operant boxes to assess whether developmental NtsR1 deletion decreases DA-mediated food intake. A) Change in body weight 24 hr after food was restored. B) Chow intake measured 1, 6, and 24 hr after chow was restored. After fasting-induced re-feeding studies, mice were tested in operant boxes. C) Depiction of operant box setup. The position of the correct nose-poke (yellow) was counterbalanced between mice. *NtsR1*$^{flox/flox}$ and DATR1^{Null} were trained to nose poke for sucrose rewards. Mice were then tested on a PR schedule for operant responding during baseline (control, *ad libitum* chow) and fasted (hungry) conditions. D) Magazine entries (where sucrose pellet is deposited), E) Correct nose pokes, F) Number of sucrose pellets obtained, G) PR value (last ratio obtained), and H) Percentage of sucrose pellets eaten. *NtsR1*$^{flox/flox}$ and DATR1^{Null} mice do not significantly differ from each other during fasting re-feeding or operant tests. Data were analyzed by Ordinary One-Way ANOVA with Sidak post-tests. Graphs represent mean ± SEM (n=9-13). $p^{*}<0.05$.

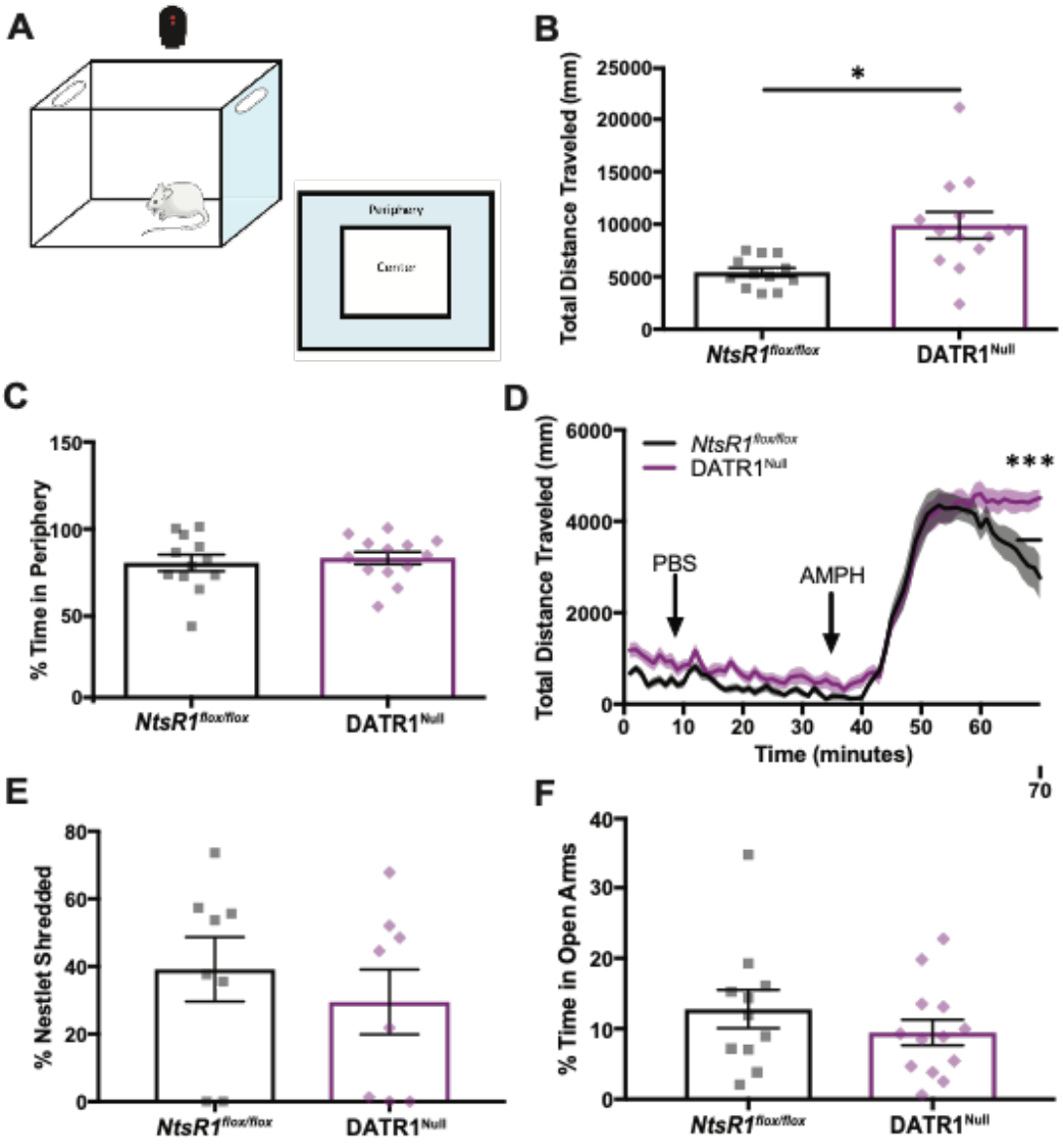

Figure 3.4 Developmental Deletion of NtsR1 Increases DA-Dependent Locomotor Activity Without Inducing Anxiety. *NtsR1$^{flox/flox}$* and DATR1^{Null} mice were placed in open field boxes and videotracked to assess exploratory behavior and locomotor activity (A). In B and C, mice were left to explore box for 30mins, bars represent last 10 minutes of data collected and analyzed. In D, mice were left to explore the box for 30 minutes after PBS injection, and for 30 minutes after amphetamine (AMPH) treatment. B) Total distance traveled under baseline condition (no

Figure 3.4 (cont'd)

injection). C) % time spent in box periphery under baseline condition. These data suggest that developmental deletion of NtsR1 from DA neurons increases the quantity of exploratory behavior but does not alter its quality. D) Total distance traveled before, during and after PBS and AMPH treatment. DATR1^{Null} mice had a significantly prolonged response to AMPH compared to *NtsR1$^{flox/flox}$* mice. To assess whether DATR1^{Null} mice engaged in anxiety-like behaviors, they were given a nestlet to shred for 30 minutes and placed in the Elevated Plus Maze (EPM) for 5 minutes. Developmental deletion of NtsR1 did not significantly alter E) % of nestlet shredding or F) % time spent in open EPM arms. Open field, nestlet shredded and EPM data represent mean ± SEM analyzed via unpaired TTEST (n=8-13). AMPH data represent mean ± SEM analyzed via ordinary 2-way ANOVA with Sidak post-tests (n=12-13). *p<0.05.

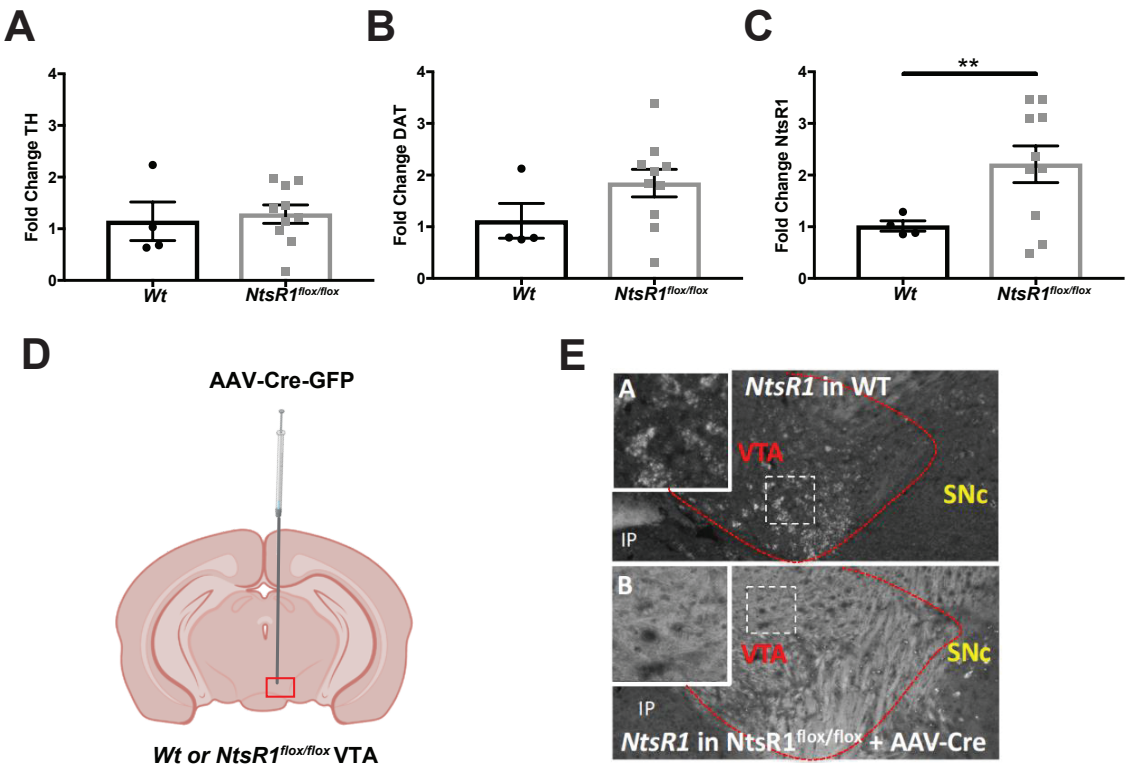

Figure 3.5 Introducing Flox Increases VTA NtsR1 Gene Expression, but AAV-Cre-GFP is Effective at Deleting VTA NtsR1. To assess whether introducing flox sequences around the NtsR1 gene induced molecular alterations in the VTA, we analyzed the fold difference in gene expression in *NtsR1*$^{flox/flox}$ and *Wt* littermates. A) *Th*, B) *Dat* and C) *Ntsr1* gene expression in the VTA of *NtsR1*$^{flox/flox}$ and *Wt* littermates. D) *NtsR1*$^{flox/flox}$ and *Wt* mice were injected unilaterally with AAV-Cre-GFP in the VTA. E-A) Representative NtsR1 mRNA-labeled images showing that NtsR1 (white) is reduced only in AAV-Cre-GFP injected *NtsR1*$^{flox/flox}$ mice. Bar graphs depict the fold change in gene of interest ± SEM. *p<0.05 via unpaired Student's t-test.

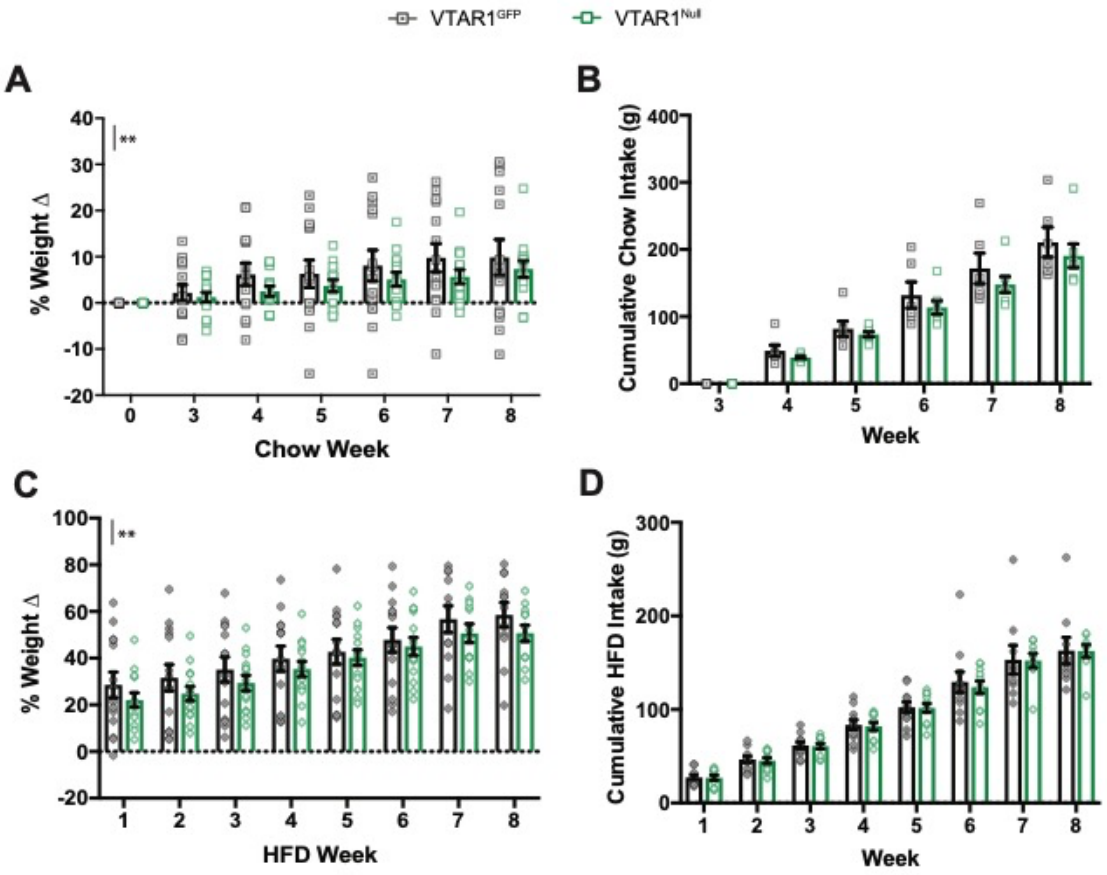

Figure 3.6 VTA Specific NtsR1 Deletion Decreases Body Weight but not Food Intake in Chow and High Fat Diet Fed Adult Mice. VTAR1^{GFP} and VTAR1^{Null} mice were individually housed right after surgery and their food and body weight was taken every week until the 8-week mark after surgery. Mice were then switched to HFD for 8 weeks. A) % Weight change from surgery day to week 8 on chow and B) cumulative chow intake. C) % Weight change from week 1-8 on HFD and D) Cumulative HFD Intake. VTAR1^{Null} mice both have lower body weights on chow and HFD than VTAR1^{GFP} mice. However, chow or HFD intake is not significantly different between chow or HFD groups. Data represent mean ± SEM analyzed via ordinary 2Way-ANOVA with Sidak's correction (n=12-13). *p<0.05.

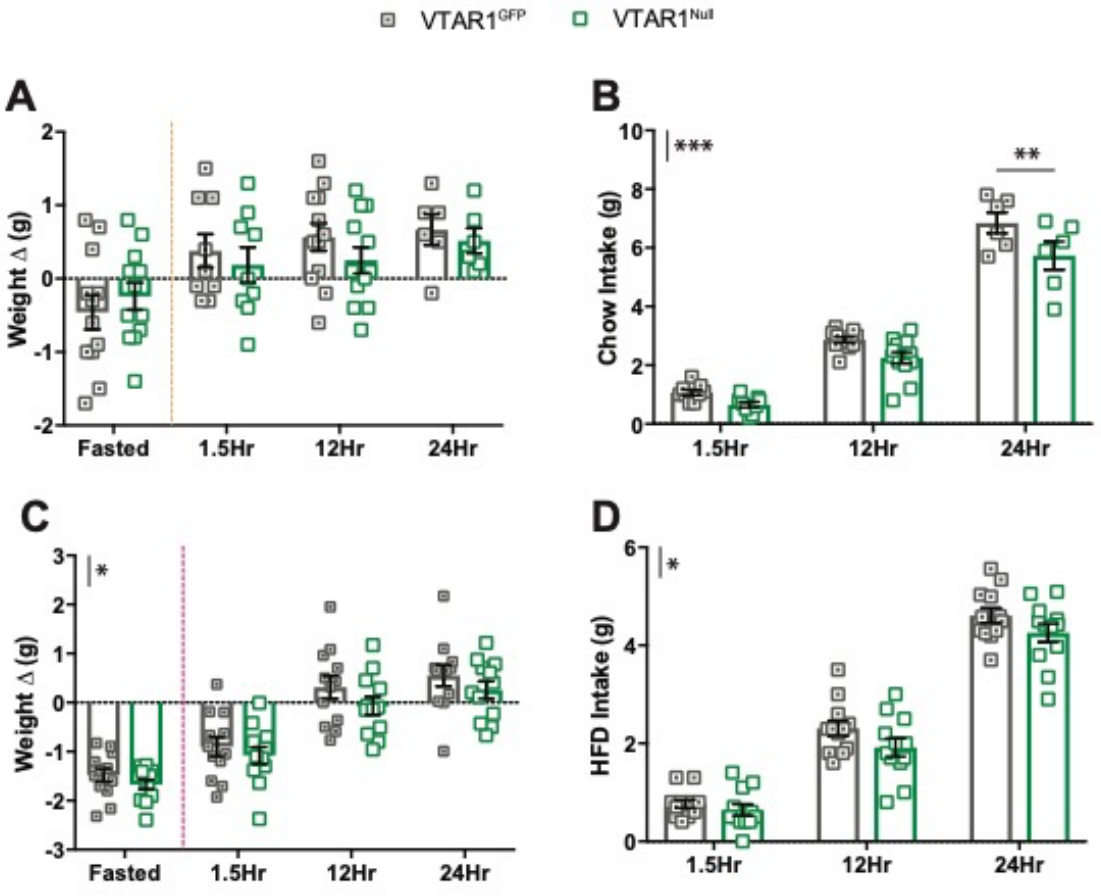

Figure 3.7 Adult VTA NtsR1 Deletion Decreases DA-Dependent Food Intake and Weight Gain.

VTAR1^{GFP} and VTAR1^{Null} mice were fasted overnight and then re-fed the next day with Chow or HFD to assess whether adult NtsR1 deletion decreases DA-mediated food intake. Weight was measured ~15 hr after mice were fasted and 1.5, 12, and 24 hours after food was restored. A) Weight change before and after normal chow was restored. B) Chow intake after chow was restored. VTAR1^{Null} mice on normal chow diet gained weight similar to VTAR1^{GFP} mice, but their chow intake was significantly reduced at 24 hr. C) Weight change before and after HFD was restored. D) HFD intake after HFD was restored. VTAR1^{Null} mice on HFD gained less weight after HFD was restored compared to VTAR1^{GFP} mice, and their HFD intake was significantly overall significantly reduced. Data represent mean ± SEM analyzed via ordinary 2Way-ANOVA with

Figure 3.7 (cont'd)

Sidak's correction for multiple comparisons. n=8-12. Mark next to the y-axis in B-D indicates overall significant differences between genotypes. *$p<0.05$.

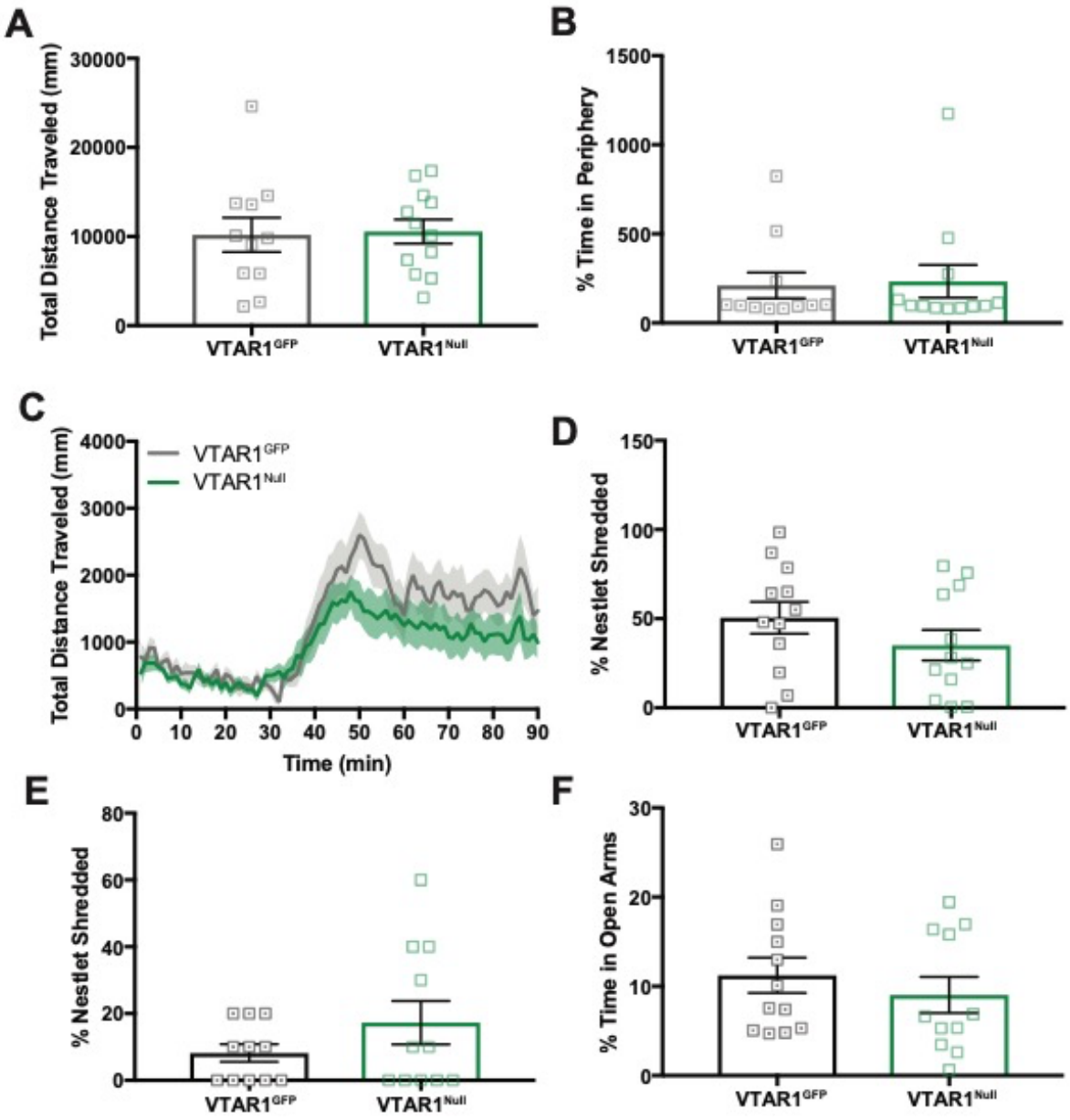

Figure 3.8 Specific VTA NtsR1 Deletion does not Alter DA-Dependent Locomotor Activity or Anxiety Behaviors. VTAR1^{GFP} and VTAR1^{Null} mice were placed in open field boxes and video-tracked to assess exploratory behavior and locomotor activity. In A and B, mice were left to explore box for 30mins, bars represent last 15 minutes of data collected and analyzed. In C, mice were left to explore the box for 30 minutes after PBS injection, and for 30 minutes after amphetamine (AMPH) treatment. A) Total distance traveled under baseline condition (no

Figure 3.8 (cont'd)

injection). B) % time spent in box periphery under baseline condition. C) Total distance traveled before, during and after PBS and AMPH treatment. VTAR1^{Null} mice did not have a significantly prolonged response to AMPH compared to VTAR1^{GFP} mice. To assess whether VTAR1^{Null} mice engaged in anxiety-like behaviors, they were given a nestlet to shred and marbles to bury for 30 minutes and placed in the Elevated Plus Maze (EPM) for 5 minutes. Deletion of NtsR1 specifically from the VTA in adulthood did not significantly alter D) % of nestlet shredding, E) % of marbles buried, or F) % time spent in open EPM arms. Open field, nestlet shredded, marbles buried and EPM data represent mean ± SEM analyzed via unpaired TTEST (n=8-13). AMPH data represent mean ± SEM analyzed via ordinary 2-way ANOVA with Sidak post-tests (n=12-13). *$p<0.05$.

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CHAPTER 4. SUMMARY, DISCUSSION, AND TRANSLATIONAL IMPLICATIONS

4.1 SUMMARY OF RESULTS

The overall goal of this PhD dissertation was to understand the role of VTA NtsR1 neurons in energy balance. The main hypothesis of the work was that *VTA NtsR1 neurons are both sufficient and necessary for weight loss behaviors that are dependent on NtsR1*.

In chapter 2, we used *NtsR1*^{Cre} mice and DREADD technology to study how experimental activation of VTA NtsR1 neurons altered energy balance in the context of normal weight and obesity. Our hypothesis for this chapter was that *targeted activation of VTA NtsR1 would recapitulate the effects of intra VTA Nts treatment (decrease feeding and increase locomotor activity), and not induce vasodepression and hypothermia*. We injected *NtsR1*^{Cre} mice in the VTA with AAVs to enable cre-mediated expression of excitatory DREADDs, thereby selectively expressing DREADDs in VTA NtsR1 neurons. To activate these neurons on command, we then systemically administered the DREADD ligand, CNO. We discovered that acute activation of VTA NtsR1 neurons was sufficient to promote dual weight loss behaviors that decreased body weight of both normal weight and diet-induced obese mice. Moreover, chronically activating VTA NtsR1 neurons in obese mice (7 days) sustained the weight loss observed during acute activation. As the majority of NtsR1 expressing neurons in the VTA are DA-ergic, we also examined whether activating VTA NtsR1 neurons altered operant responding for palatable foods, led to increased anxiety, and/or addiction behaviors. We found that activating VTA NtsR1

neurons reduced feeding and operant responding for palatable food, but we did not augment anxiety behaviors or place preference. We also found that acute activation of VTA NtsR1 neurons did not result in adverse cardiorespiratory effects or hypothermia, as have been observed in rodents treated with Nts systemically or in the hindbrain[1–4]. These results highlight the importance of neuropeptide receptors in energy balance and suggest that VTA NtsR1 neurons may be a biologic target that could be leveraged to treat obesity.

In chapter 3, we assessed whether NtsR1 expression in DA neurons was required for weight loss. We reasoned that if activation of VTA NtsR1 neurons decreased feeding and increased locomotor activity to support weight loss, then specific deletion of NtsR1 from VTA DA neurons would increase feeding, reduce locomotor activity, and increase body weight. For these studies, we used *NtsR1*$^{flox/flox}$ mice. Notably, NtsR1 is transiently expressed throughout the brain during early development, but expression is restricted in the adult brain to precise regions, and is particularly concentrated within the VTA[5–9]. This temporal expression of NtsR1 suggests that it may contribute differently to physiology during development than in the established nervous system, hence, we elected to study the requirement for NtsR1 in both settings. We used *NtsR1*$^{flox/flox}$ mice to generate both a developmental and an adult-onset model for NtsR1 deletion from DA neurons. We discovered that developmental deletion of NtsR1 from all DA-expressing neurons (developmental model) has no impact on metabolism, body weight or food intake. Interestingly, developmental deletion of NtsR1 increases locomotor activity, but only when the mice are exposed to a novel environment. We also found that developmental NtsR1 deletion did not induce anxiety behaviors. To assess the integrity of the

mesolimbic DA system, we tested developmental model mice in operant chambers and treated them with amphetamine. We did not find any significant differences between mice with developmental deletion of NtsR1 and their *Wt* littermates in willingness to work for a reward. However, we did find that they sustained increased amphetamine-induced locomotor activity, which could indicate that deletion of the receptor leads to enhanced DA signaling in some contexts. These results suggest that developmental deletion of NtsR1 from all DA-expressing neurons at an early stage may invoke organizational and compensatory changes that offset the impact of lacking NtsR1 for normal physiology. Thus, although VTA NtsR1 neuronal activation was sufficient to alter the DA system and energy balance, developmental expression of NtsR1 in DA-expressing neurons is not necessary for execution of DA-mediated behavior and regulation of body weight.

In chapter 3, we also examined whether deletion of NtsR1 only from VTA neurons in adulthood (adult model) deranged energy balance. We combined site-directed viral injections into the VTA of adult *NtsR1*$^{flox/flox}$ mice to selectively delete NtsR1 from established VTA NtsR1-DA neurons, then provided them with normal chow and HFD to assess the contribution of food type consumed in altering the NtsR1 system and its relation to energy balance. When NtsR1 is deleted from VTA neurons in adulthood we found that mice both on normal chow and HFD, had lower body weight, but this was not explained by a decrease in food intake or an increase in exploratory locomotor activity. However, deleting NtsR1 from VTA neurons in adulthood does reduce the amount of normal chow and HFD mice will consume after being fasted, which results in overall decreased weight gain in HFD mice 24hrs after food is returned. As observed

with developmental deletion, we did not find that adult deletion of the receptor induced anxiety behaviors. In contrast to developmental deletion however, amphetamine-induced locomotor activity was also not altered in adult model. Altogether, these data suggest that loss NtsR1 expression does not promote weight gain, and in fact, that reducing NtsR1 expression in the adult VTA may be useful for weight loss.

4.2 DISCUSSION

4.2.1 *Limitations and Technical Considerations of this work*

We leveraged viral and cre-lox mediated tools throughout this dissertation to specifically modulate VTA NtsR1 neurons and test our hypotheses. Therefore, our results are limited by the efficiency of the mouse models and viral tools used in these experiments. To address potential caveats of these reagents we used several controls for each mouse strain, virally mediated tool and technique that modulated gene expression or neuronal activity.

For the experiments in chapter 2, we used a knock in *NtsR1*^{Cre} model because knock-in lines are reliable reporters of endogenous gene expression, particularly for lowly-expressed transcripts such as receptors[10,11]. However, it is recognized that introducing IRES Cre after the stop codon (as is the case in *NtsR1*^{Cre} mice) can sometimes influence expression of the upstream coding sequence[12]. To control for this possibility, we used a within-subjects approach, so that *NtsR1*^{Cre} mice could be used as their own controls.

We also used well-established viral tools for chemogenetic studies (DREADDs) to modify the activity of VTA NtsR1 neurons and understand their function in energy balance. Even though DREADDs have been enormously useful to dissect how specific neuronal populations contribute to physiology and behavior, there are still limitations in using them. The DREADD ligand CNO has been reported to be inert, but it may have off target effects due to the actions of its metabolite, clozapine. Therefore, it was important to verify that the CNO dosage used for our chemogenetic studies does not exert any effects when injected into non-DREADD expressing mice. For this reason, we included non cre-expressing *Wt* controls in this study that received the same CNO treatments as DREADD-expressing *NtsR1*^{Cre} mice. We also used a low concentration of CNO (0.3 mg/kg) that does not produce off-target effects in contrast to higher concentrations[13]. These controls suggested that CNO has no significant off-target effects on energy balance behaviors in mice lacking DREADD receptors, consistent with previous reports from our lab[14,15]. Thus, the CNO-treatment induced alterations in behaviors reported in this dissertation represent genuine physiological responses.

Another caveat of using DREADDs to study neuronal mediated behavior is that chemogenetic-mediated neuronal activation does not guarantee that we have activated the same intracellular mechanism in the neuron that would be engaged by Nts binding to NtsR1. The excitatory DREADD receptors used here and NtsR1 are both Gq-coupled GPCRs. NtsR1 has been thoroughly researched for its Gq-coupling; however, recent structural analysis of NtsR1 suggests that it also couples with β-arrestins that regulate downstream cellular events[16]. This is because β-arrestins are important in suppressing G-protein signaling for most GPCRs (the

interaction of GPCRs with β-arrestins represents a desensitized or inactive G-protein complex)[16]. In the future, studies using NtsR1 biased agonists could further connect behaviors to particular intracellular mechanisms of NtsR1 activation, and could perhaps help to dissect whether there are distinct contributions of the Gq vs. β-arrestin signaling pathways to Nts-NtsR1 mediated physiology[17].

One unique aspect of our work in Chapter 3 was assessing whether activating VTA NtsR1 neurons impacted feeding as well as cardiovascular regulation. Although Nts-NtsR1 signaling has been implicated in regulating both of these aspects of physiology, they are typically not studied together or within the same models. Some peptides produced in the brain that regulate food intake and energy expenditure are also involved in blood pressure regulation. Indeed, brain mechanisms can play a significant role in both the regulation of food intake, energy expenditure and short- and long-term blood pressure control. The hypothalamus and brainstem seem to be the major structures in the regulation of both food intake and blood pressure, and the hypothalamus is where the actions of many peptides and neurotransmitters are integrated to mediate dietary intake. Moreover, both anorexigenic and orexigenic peptides have been shown to increase or decrease blood pressure. However, studies about the effect of food intake regulating peptides and blood pressure use different animal models and routes of peptide administration (central vs. peripheral), which has hindered appreciation of whether feeding and blood pressure are regulated by common or separate systems. This is a limitation in the Nts-NtsR1 field, as it is well known that Nts exerts different effects when administered in the periphery vs. the brain[18,19]. Nts is found in cardiovascular tissue, and recent clinical studies have

highlighted the contribution of Nts-mediated mechanisms in pathogenesis of various cardiovascular conditions. It has been shown that systemic Nts levels are elevated in patients with some circulatory disorders[20,21], and increased plasma concentrations of the Nts protein precursor, proneurotensin, are associated with increased risk of cardiovascular morbidity and mortality[22]. Moreover, Nts-induced cardiovascular responses are primarily mediated via activation of NtsR1[23]. NtsR1 is expressed in rat myocardial tissue[24] and in human umbilical vein and aortic endothelial cells[25] suggesting it can mediate direct regulation of cardiovascular activity. The data on NtsR1's role in cardiovascular regulation, however, is equivocal, as it has been shown to elicit hypotensive or hypertensive effects depending on the animal model used in the study[26–35]. In rats, the most widely reported effects of systemic treatment with Nts or NtsR1 agonists are hypotension and decreased core body temperature[1–4,36]. We found that specifically activating VTA NtsR1 neurons promoted weight loss behaviors but did not make mice hypothermic or lower their blood pressure and that VTA NtsR1 neural activation during the light cycle did not reduce, but rather increased mean arterial pressure and heart rate. While chemogentic activation of VTA NtsR1 neurons is not the same as Nts binding to NtsR1, our data hint that signaling VTA NtsR1 neurons might bias for regulation of feeding behavior rather than blood pressure. If true, this would identify VTA NtsR1 neurons as a potential target for modulating feeding and body weight without causing potentially dangerous disruptions to thermoregulatory and cardiovascular systems.

In chapter 3, we used a newly generated *NtsR1*$^{flox/flox}$ mouse to explore the requirement for NtsR1 in DA neurons and whether it may differ throughout lifespan. As explained above

with the use of knock-in *NtsR1*^{Cre} mice, *NtsR1*$^{flox/flox}$ mice may not be physiologically identical to *Wt* mice because we have introduced alterations into its genomic DNA[37]. Indeed, we found that introducing loxP sites flanking exon 1 of *Ntsr1* increased NtsR1 gene expression compared to *Wt* littermates. A caveat of this study is that increased protein expression of NtsR1 could lead to differences in baseline behaviors regulated via NtsR1. Due to the lack of reagents to measure NtsR1 protein we cannot verify that the increased mRNA equates to elevated NtsR1 protein. In lieu of this we queried for behavioral differences but did not observe any significant differences between *NtsR1*$^{flox/flox}$ and *Wt* genotypes (data not shown), suggesting that the elevated *Ntsr1* mRNA is not disrupting normal physiology. However, to control for any potential issues we limited our studies to inter-comparing the effect of cre-mediated NtsR1 deletion or intact NtsR1 expression within the *NtsR1*$^{flox/flox}$ strain.

Germline conditional deletion of NtsR1 from DA neurons (the developmental model) produced only subtle behavioral changes. This is not completely surprising, as conditional germline deletion of many genes is known to impart compensatory regulation that masks the true necessity of the conditionally deleted gene. We posit that this may have occurred with our developmental deletion of NtsR1 from DA neurons, as it did not alter appetitive behavior but only modulated DA-mediated arousal/attention behaviors (e.g., novel object and AMPH responding). In our adult model mice, we also only observed a modest phenotype for weight loss, and we reasoned that deleting a single GPCR may indeed produce a modest phenotype. However, the impairments in body weight and fasting-induced refeeding observed after adult-

onset NtsR1 deletion in the VTA suggests that NtsR1 plays a role in tuning appropriate energy intake behavior and body weight.

For all experiments in this dissertation, we have included male and female mice since we recognize that it is important to investigate potential sex-specific regulation of feeding, locomotor activity and weight loss via VTA NtsR1 circuits. Although we observed some trends in our data, particularly with developmental deletion of NtsR1, we did not find any significant differences between sexes. Deletion of NtsR1 presented a mild weight loss phenotype in our study mice, but we did not find that this phenotype was biased towards either sex. Since we did not detect any statistical differences between male and female mice, data obtained from both sexes were pooled for analysis.

4.2.2 ***Future Directions***

The culmination of this dissertation shows that VTA NtsR1 neurons play a role in weight loss. Although this work provides preliminary evidence that NtsR1 signaling may be a candidate for treating obesity, several questions and challenges need to be addressed to fully understand the function of NtsR1 in energy balance. Notably, our studies and the majority of those in the field have been restricted to rodent models. To truly appreciate the clinical potential of the Nts-NtsR1 system for modulating energy balance it will be necessary to evaluate the expression and distribution levels of NtsR1 in humans, and see if they are consistent with what has been observed in animal modles. Clearly more work with human tissue is necessary to confirm the

nature of the NtsR1 system.

NtsR1 agonists and antagonists have been used to identify the role of the receptor in regulating behaviors that affect body weight. NtsR1 agonists suppress feeding[3] and NtsR1 antagonists block the anorectic and locomotor effects of Nts in adult rodents[15,38,39]. Thus, it is feasible to pharmacologically modulate the NtsR1 system. However, systemic or central agonism of the receptor could bind to NtsR1 in tissue that does not exclusively modulate weight loss behaviors[1–4]. Therefore, the potential for this system for treating obesity depends on whether NtsR1 agonists can selectivety promote activity only in VTA NtsR1 neurons. Another limitation of pharmacologically modulating the system is that we do not fully understand the intracellular mechanism of NtsR1 action. There are reports of NtsR1 coupling to Gq and β-arrestins (which promote receptor internalization), as well as the existence of NtsR1-Dopamine Receptor-2 heterodimers[40]. Thus, it may be possible to design biased agonists that might at least confine the drug to a specific signaling pathway and/or the DA system. Whether such mechanisms are targetable *in vivo* is another question that must be resolved to assess the utility of modulating VTA NtsR1 neurons. Answering these questions is important to understand the mechanism by which NtsR1 contributes to energy balance, and how its signaling might be modulated to bias for weight loss. Alternatively, better understanding of NtsR1-mediated signaling may identify strategies to promote beneficial behaviors without adverse physiological effects.

Low dopaminergic activity is associated with obesity in humans. Individuals who receive DA receptor antagonists are at an increased risk of weight gain, and administration of D2

receptor agonists in rats results in reduced body weight[41,42]. Thus, activating DA neurons may be a potential strategy to support weight loss. However, DA signaling can promote anxiety, aversion or reinforcement, and these undesirable psychiatric effects would outweigh any beneficial effects of body weight regulation[43,44]. Notably, we did not find that activating VTA NtsR1 neurons induced these effects. This is likely due to the specificity of our manipulations and system, since we selectively modulated VTA NtsR1 neurons that are a subpopulation of all DA neurons. Going forward, it is vital to define the impact of activating NtsR1 protein within the VTA specficially, rather than activating the VTA NtsR1 neurons , so as to separate the effects of NtsR1 and DA in energy balance. Our finding that lack of NtsR1 in DA neurons increased novel environment locomotor activity and prolonged increased AMPH induced activity suggest that developmentally deleting NtsR1 from DA neurons may lead to enhanced DA signaling. Numerous mechanisms could enhance DA action, such as impaired DAT kinetics or altered balance of D1/D2 expression that is thought to underlie the hyperdopaminergic phenotype of constitutive NtsR1 knockout mice. It is important to also note that crossing *NtsR1*$^{flox/flox}$ mice to *DAT*^{Cre} leads to cre-mediated deletion germline deletion of NtsR1 from all DA neurons, including those of the VTA and substantia nigra. This could explain why our developmentally deleted mice have increased AMPH responding and exploratory behaviors, as DA arising from the substantia nigra may be more related to attention processes while DA from the VTA is more important for reward processing[45]. Thus, while there is much yet to be learned about the biology of NtsR1 in mediating energy balance, our data support further exploration of VTA NtsR1 neurons as potential pharmacological targets to improve the treatment of obesity and its co-morbidities.

4.3 TRANSLATIONAL IMPLICATIONS OF THIS RESEARCH

VTA DA neurons have been linked to energy balance before, but how they mediate these behaviors remains unclear. This is in part due to the heterogeneity of cells in the VTA, and its projections to multiple brain regions. Defining and understanding the various types of VTA circuits and their contribution to normal physiology, as well as how disruptions contribute to disease pathogenesis could help identify treatments for energy balance disorders. However, these circuits have primarily been studied in rodent models, largely due to the powerful array of Cre/Lox mouse lines and site-specific tools that permit reserachers to identify and manipulate specific neurons and circuitry. Such mechanistic studies of the VTA are simply not possible in humans. Studies of the human VTA have been limited to imaging studies, which lack the cellular resolution of rodent studies, but provide information about the role of the VTA in energy balance behaviors[46,47]. Even though there are not yet as many human studies about the role of the VTA in energy balance, these studies still support decades of accumulated results from rodents, signifying the translational value of rodent studies to decipher the central circuits controlling energy balance behaviors.

Similarly, the role of NtsR1 and its contributions have also been largely studied in rodent systems to date, with much less known about NtsR1 regulation in humans. There are a few studies about Nts- and NtsR1-body weight relation in humans, including implication of a role for NtsR1 mutations in eating disorders[48–50]. In addition, NtsR1 and Nts gene mutations have also been found in schizophrenic patients, although no causative association with the disease have been identified to date[51,52]. While genetic mutations or deltions of NtsR1 have yet to be

characterized and linked as causing disease, this does not mean that NtsR1 does not contribute to disease states. Altered NtsR1 function could also arise from changes in the concentration of available Nts ligand, biased intracellular signaling pathways or other epigenetic changes that would not be appreciable via genome wide association studies. Thus, there is much yet to be learned from experimental models about how NtsR1 is altered in disease, which may ultimately provide insight about the role of NtsR1 in human physiology.

The basic science work of this dissertation, while confined to rodents, advances understanding of the physiological control of feeding and locomotor behaviors that promote weight loss, and could have implications for treatment of disrupted energy balance behaviors in humans. Our finding that the activation of VTA NtsR1 neurons in normal weight and obese mice are sufficient to promote weight loss refines the field's knowledge of how weight loss is mediated, and what circuitry might be targeted to modify energy balance. We showed selective activation of VTA NtsR1 neurons decreases the motivation to work for rewards, but not enough to disrupt baseline homeostatic and need-based consumption. Moreover, we showed that actvating NtsR1 neurons promotes weight loss behaviors without inducing anxiety, preference, or adverse cardiovascular effects. We also showed that specifically deleting NtsR1 from VTA neurons in adulthood promotes lower bdy weight. From a translational perspective, these results may be relevant in treating obesity and its co-morbidities, as activating VTA NtsR1 promotes dual weight loss behaviors.

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