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

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SUMMARY

Dopamine (DA) neurons in the ventral tegmental area (VTA) are heterogeneous and differentially regulate ingestive and locomotor behaviors that impact energy balance. Identification of which VTA DA neurons mediate behaviors that limit weight gain has been hindered, however, by the lack of molecular markers to distinguish VTA DA populations. Here, we identified a specific subset of VTA DA neurons that express neurotensin receptor-1 (NtsR1) and preferentially comprise mesolimbic, but not mesocortical, DA neurons. Genetically targeted ablation of VTA NtsR1 neurons uncouples motivated feeding and physical activity, biasing behavior toward energy expenditure and protecting mice from age-related and diet-induced weight gain. VTA NtsR1 neurons thus represent a molecularly-defined subset of DA neurons that are essential for the coordination of energy balance. Modulation of VTA NtsR1 neurons may therefore be useful to promote behaviors that prevent the development of obesity.

eTOC BLURB

Woodworth et al. identify a subset of VTA dopamine neurons that express neurotensin receptor-1. Ablation of these neurons leads to enhanced physical activity and energy expenditure that protects mice from diet-induced obesity, revealing an important role for VTA NtR1 neurons in the regulation of body weight.

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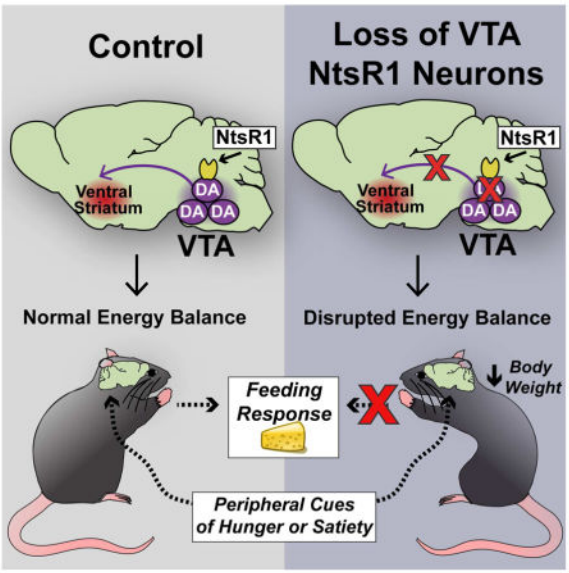
AUTHOR CONTRIBUTIONS

HLW and GML designed the experiments and wrote the paper. HLW conducted the experiments and analyzed the data, with assistance from HMB, BB, RB, JAB, and GK. PMF provided AAV-DTA and read the manuscript.



Keywords

Neurotensin Receptor; Dopamine; Nucleus Accumbens; Locomotor Activity; Feeding; Body Weight; Metabolism; Obesity; Mesolimbic

Obesity is caused by overconsumption of calorie-dense food combined with insufficient physical activity, and predisposes individuals to myriad chronic conditions that shorten life span. Proper diet and exercise are important for maintaining healthy weight but these lifestyle modifications are difficult to maintain long-term. Consequently, most individuals that lose weight through changes in diet and activity tend to regain it over time (Soleymani et al., 2016). Therapeutic strategies to suppress motivated feeding and encourage physical activity would therefore be helpful to prevent weight gain and combat the obesity epidemic.

The motivated feeding and locomotor behaviors that influence body weight are regulated, in part, by dopamine (DA) neurons in the ventral tegmental area (VTA) that release DA to the ventral striatum and prefrontal cortex (PFC) (Day et al., 2007; McCullough et al., 1993). DA itself is essential for energy balance, demonstrated by the aphagia, hypolocomotion, reduced body weight, and early lethality of mice that genetically lack DA (Zhou and Palmiter, 1995). Disruptions in DA signaling are also observed in obese rodents and humans, suggesting that inappropriate regulation of DA circuits contributes to weight gain (Beeler et al., 2015; Johnson and Kenny, 2010; Stice et al., 2008). Yet, the mechanisms by which VTA DA neurons orchestrate energy balance remain poorly understood. One emerging theory is that VTA DA neurons are not homogenous, but in fact consist of subsets of neurons that coordinate distinct aspects of feeding and locomotor activity. Indeed, VTA DA neurons can be differentiated according to their electrophysiological firing properties (Lammel et al., 2008) or their anatomical inputs and projections (Lammel et al., 2011; Lammel et al., 2012). Mesolimbic VTA DA neurons project to the ventral striatum (consisting of the nucleus accumbens [NA] and olfactory tubercle [OFT]) and are primarily activated by “rewarding” stimuli, whereas mesocortical DA neurons project to the PFC and are regulated by

"aversive" cues (Lammel et al., 2011). Some VTA DA neurons also contain the classical neurotransmitters GABA or glutamate (Root et al., 2014; Stamatakis et al., 2013) that can promote or suppress feeding in the NA (Ho and Berridge, 2014; Reynolds and Berridge, 2003). Collectively, these data suggest the possibility that distinct VTA DA populations modify obesogenic behaviors. Unfortunately, no criteria to date identify which specific VTA DA populations may be leveraged to treat energy balance disorders. Molecular markers to differentiate subsets of VTA DA neurons would be useful in this regard. While the transcription factor *Neurod6* distinguishes the subset of lateral septum-projecting VTA DA neurons (Khan et al., 2017), markers for mesolimbic or mesocortical VTA subsets remain to be identified.

Neuropeptides such as orexin-A/B, neuropeptide-Y, corticotropin releasing factor (CRF), glucagon-like peptide-1 (GLP-1), and neurotensin (Nts) differentially modify DA-dependent behaviors (Anderberg et al., 2014; Kalivas et al., 1983; Vittoz and Berridge, 2006; Wanat et al., 2008), thus we reasoned that VTA DA neurons might be distinguished by their expression of neuropeptide receptors. Of these systems, Nts holds particular promise for modifying obesogenic behaviors since pharmacologic administration of Nts in the VTA restrains feeding, increases locomotor activity and induces weight loss in obese rodents (Cador et al., 1986; Elliott and Nemeroff, 1986; Feifel et al., 2010). Nts signals via neurotensin receptors -1 and -2 (NtsR1 and NtsR2) that are expressed in the VTA (Lepee-Lorgeoux et al., 1999; Palacios et al., 1988), and Nts enhances the activation of VTA DA neurons and DA release in the NA (Kalivas et al., 1983; Sotty et al., 1998). The lack of reagents to identify cells expressing NtsRs, however, has hampered understanding of how Nts mechanistically engages DA neurons. To overcome this challenge, we used Cre-lox technology to selectively visualize NtsR1 or NtsR2-expressing cells in mice and identified a unique subset of VTA DA neurons that express NtsR1. Disrupting these VTA NtsR1 neurons alters DA-mediated behaviors to prevent weight gain, suggesting that this subset of VTA neurons may represent a cellular target for preventing the development of obesity.

RESULTS

NtsR1 and NtsR2 Identify Distinct VTA Cell Types

We crossed existing transgenic *NtsR1*^{Cre} mice and newly generated knock-in *NtsR2*^{Cre} mice onto a Cre-inducible-GFP reporter line, such that progeny expressed GFP selectively in NtsR1 or -2 expressing cells, respectively (Fig. S1, S2A). These models revealed striking differences in the morphology, number and distribution of NtsR1-GFP and NtsR2-GFP cells throughout the rostro-caudal axis of the VTA (Fig. 1A). While a few NtsR2-GFP cells appeared to be neurons (Fig. 1A white arrows), the vast majority were stellate-shaped cells consistent with glial morphology (Fig. 1A yellow arrows). By contrast, NtsR1-GFP cells were exclusively neuronal (Fig. 1A) and more numerous than NtsR2-GFP neurons. The NtsR1-GFP neurons were predominantly located in the rostral VTA (bregma-3.40 to -3.05), while the few NtsR2-GFP neurons clustered along the ventral-medial border of the caudal VTA (bregma -3.64), a region enriched in GABAergic neurons (Mazei-Robison and Nestler, 2012).

Given the distinct morphology and distribution of NtsR1-GFP and NtsR2-GFP cells, we next used immunolabeling to determine if they represent distinct cellular populations. The NtsR2-GFP cells with stellate morphology co-localized with the astrocyte marker S100, confirming that most NtsR2 cells are glia (Fig. 1B, yellow arrows). By contrast, none of the NtsR1-GFP cells or the few NtsR2-GFP cells with neuronal morphology co-localized with S100 (Fig. 1B, C). Instead >90% of NtsR1-GFP neurons co-expressed tyrosine hydroxylase (TH), a marker of DA neurons (Fig 1. E–H), consistent with our previous findings (Opland et al., 2013). Only ~30% of the much smaller population of NtsR2-GFP neurons co-expressed TH, representing ≤1% of all VTA DAergic neurons (Fig. 1D, F–H). Collectively, these data reveal that NtsR1 and NtsR2 are expressed in non-overlapping VTA subpopulations, that NtsR1 is the predominant receptor isoform expressed on VTA neurons, and that VTA NtsR1 neurons are a subset of all VTA DA neurons.

VTA NtsR1 Neurons are a Projection-Defined Subset of VTA DA Neurons

We next investigated whether the subset of VTA NtsR1 neurons are a mesolimbic- or mesocortical-specified population of VTA DA neurons by injecting the Cre-mediated anterograde tract tracer Ad-Syn-mCherry (Opland et al., 2013) into the VTA of *NtsR1*^{Cre} mice (Fig. 2A, B, Fig. S2B–D). This method revealed that VTA NtsR1 neurons densely project to the ventral striatum, including the NA core and shell (NAc and NAs) and the olfactory tubercle (OFT) (Fig. 2C, E). Numerous VTA NtsR1 terminals were also observed within NA-adjacent regions such as the interstitial nucleus of the posterior limb of the anterior commissure (IPAC, Fig. 2C, E) (Heimer et al., 1997) and the ventral caudate-putamen (CPu) (Fig. 2C, E). There was, however, a notable absence of VTA NtsR1 terminals in the PFC (Fig. 2C, E). We also examined other projection targets of the VTA, and observed minor terminals within the caudal CPu and basolateral amygdala (BLA) but none within the central amygdala (CeA) or the hippocampus (Hipp) (Fig. 2D). Thus, NtsR1 defines a subset of VTA DA neurons that predominantly project to the ventral striatum and contiguous regions rather than the PFC. This indicates that VTA NtsR1 neurons preferentially define a subset of mesolimbic, not mesocortical VTA DA neurons.

Loss of VTA NtsR1 Neurons Alters Energy Balance to Promote Leanness

To determine if VTA NtsR1 neurons are required for the control of energy balance, we genetically lesioned them (Pei et al., 2014) (Fig. 3A). Adult *NtsR1*^{Cre}*;GFP* mice received bilateral injections of an adeno-associated virus (AAV) expressing Cre-dependent Diphtheria Toxin A subunit (AAV-DTA) into the VTA, and DTA expression selectively kills VTA NtsR1 neurons (*NtsR1*^{DTA} mice). Neighboring cells lacking Cre remain intact since they neither express the toxic DTA nor the B subunit required to internalize it. Separate *NtsR1*^{Cre}*;GFP* mice received bilateral VTA injections of AAV-GFP and retained intact NtsR1 neurons (*NtsR1*^{GFP} controls). We verified that AAV-DTA sufficiently depleted VTA NtsR1 neurons as early as two weeks post-surgery, but the remaining TH-positive DA neurons confirmed that this method does not ablate DA neurons that do not express NtsR1 (Fig. 3B). We therefore compared *NtsR1*^{DTA} mice and controls to reveal the role of VTA NtsR1 neurons in energy balance. *NtsR1*^{DTA} mice exhibited significantly reduced body weight compared to *NtsR1*^{GFP} controls by 4 wks post-surgery and remained leaner (Fig. 3C) with decreased body fat percentage (Fig. 3D). Surprisingly, *NtsR1*^{DTA} mice consumed

increasingly more chow (Fig. 3E) and drank more water over the study compared to *NtsR1*^{GFP} controls (Fig. S3A), suggesting that loss of VTA NtsR1 neurons promotes ingestive behavior. While the increased energy intake of *NtsR1*^{DTA} mice was surprising given their decreased body weight (Fig. 3I, J), we wondered if augmented energy expenditure might explain why these mice remain lean. To this end, we noted hyperactivity of *NtsR1*^{DTA} mice in their home cages (Video S1, S2), and metabolic analysis confirmed increased locomotor activity compared to *NtsR1*^{GFP} controls (Fig. 3F). Increased locomotion generally demands a commensurate increase in respiration to support the activity, and indeed the hyperlocomotive *NtsR1*^{DTA} mice had increased VO_2 and VCO_2 (Supp. Fig. 3C, D and F, G) and exhibited increasingly higher rates of energy expenditure over the course of the study compared to controls (Fig. 3H, J). The respiratory exchange ratio (RER) was unaffected (Fig. S3E, H) indicating that loss of VTA NtsR1 neurons does not alter energy substrate usage. Collectively, these data indicate that loss of VTA NtsR1 neurons biases energy balance toward increased physical activity and energy expenditure to promote leanness.

Given the lean phenotype of mice lacking VTA NtsR1 on a normal chow diet, we hypothesized that they would be protected from diet-induced obesity. *NtsR1*^{DTA} mice gained significantly less weight on high fat (HF) diet and were completely protected from diet-induced increase in body fat percentage compared to control mice (Fig. 3K, L), which persisted for at least 10–13 months post-surgery (Fig. S3O). Although *NtsR1*^{DTA} mice consumed more HF diet (Fig. 3M) and had higher weight-adjusted energy intake than *NtsR1*^{GFP} controls (Fig. 3O, Q), they also exhibited escalating increases in locomotor activity (Fig. 3N), VO_2, VCO_2 (Figure S3 I, J and L, M) and energy expenditure (Fig. 3P, R) over the study. Thus, *NtsR1*^{DTA} mice remain lean in spite of their obesogenic diet, and this was due to chronically increased locomotor activity and energy expenditure.

Loss of VTA NtsR1 Neurons Alters Hedonic and Motivated Sucrose Intake

Loss of VTA NtsR1 neurons could also promote leanness by reducing the hedonic value of food, leading to insufficient caloric intake needed to balance increased energy expenditure. Surprisingly, *NtsR1*^{DTA} mice preferred and over-consumed sucrose compared to control mice (Fig. 4A–C). We reasoned that if *NtsR1*^{DTA} over-consume sucrose to try and meet homeostatic need, then their intake should be suppressed by treatment with the appetite-suppressing hormone, leptin (Halaas et al., 1995). While leptin mildly diminished sucrose preference in *NtsR1*^{GFP} controls, it did not suppress the sucrose preference of *NtsR1*^{DTA} mice (Fig. 4D). These data suggest that loss of VTA NtsR1 neurons prevents appropriate response to leptin, compromising the ability to adapt caloric intake and locomotor behaviors as needed to maintain normal weight.

We next examined whether loss of VTA NtsR1 neurons might have uncoupled motivated feeding required to offset increased energy expenditure. Despite being hyperactive, *NtsR1*^{DTA} mice correctly learned to self-administer sucrose in a time frame similar to that of control mice (Fig. 4E, H), confirming that loss of VTA NtsR1 neurons does not compromise learning or attention processes required for motivated intake. Furthermore, *NtsR1*^{DTA} and *NtsR1*^{GFP} mice worked equally to obtain sucrose (Fig. 4I, J), thus loss of VTA NtsR1

neurons does not prevent sucrose “wanting”. Given the extreme energy demands of *NtsR1*^{DTA} mice, however, their lack of increased motivated responding is counterintuitive: these mice should be more motivated than controls to consume calories (Fulton et al., 2000). We therefore reasoned that VTA NtsR1 neurons may be required to coordinate signals of energy status with appropriate adaptations in motivated behavior needed to maintain body weight. If this were true, then *NtsR1*^{DTA} mice lacking VTA NtsR1 neurons should be unable to adjust their motivated intake in response to peripheral energy cues. As expected, *NtsR1*^{GFP} control mice were less motivated to nose-poke for sucrose in response to cues of energy sufficiency such as sucrose pre-feeding (Scheggi et al., 2013) or leptin (Sharma et al., 2012), but *NtsR1*^{DTA} mice did not adjust their intake in response to these cues (Fig. 4K, L). Taken together, these findings reveal an important role for VTA NtsR1 neurons in linking metabolic status and motivated feeding necessary to maintain body weight.

Loss of VTA NtsR1 Neurons Modifies DA-Mediated Locomotor Activity

The hyperactivity of *NtsR1*^{DTA} mice suggested that they retained DA-mediated locomotor behavior. Consistent with this, open field locomotor activity of *NtsR1*^{DTA} and *NtsR1*^{GFP} mice was not disrupted by PBS-injection stress (Fig. 5A, B, Test A), whereas treatment with the D1 receptor antagonist SCH23390 (0.1mg/kg *ip*) blunted locomotor activity in both groups (Fig. 5A, B, Test B). We therefore used amphetamine (AMPH)-induced locomotor activity to assess the integrity of the mesolimbic DA system in *NtsR1*^{DTA} mice. As expected, AMPH treatment (4mg/kg *ip*) significantly increased locomotor activity in *NtsR1*^{GFP} mice, but *NtsR1*^{DTA} mice did not respond with increased activity (Fig 5D–G). Instead, we anecdotally observed that AMPH-treated *NtsR1*^{DTA} mice engaged in stationary, stereotypic movements typical with ceiling effects of DA signaling, which account for their apparent decrease in ambulatory activity (Fig. 5D–G). Since *NtsR1*^{DTA} mice lack a subset of VTA DA neurons that projects heavily to the NA, we examined the ability of AMPH to induce DA release in the NA using AMPH-induced cFos immunoreactivity to identify activated NA neurons. AMPH treatment significantly increased numbers of cFos-positive cells in the NA of both *NtsR1*^{DTA} and control mice, although the response was non-significantly blunted in *NtsR1*^{DTA} mice, consistent with loss of some, but not all NA-projecting VTA DA neurons (Fig. 5H, I). Together, these data indicate that loss of VTA NtsR1 neurons does not completely abolish mesolimbic signaling. In fact, the high baseline locomotor activity and AMPH-induced stereotypy observed in *NtsR1*^{DTA} mice may indicate enhanced DA signaling, similar to compensatory increases in extracellular DA that occur with partial loss of DA neurons (Robinson et al., 1994). Importantly, although hyperactivity and excessive DA have been linked with increased anxiety states, there were no differences in anxiety-like behavior between *NtsR1*^{GFP} and *NtsR1*^{DTA} mice as assessed via open field center activity (Fig. 5C, F) and elevated plus maze (EPM) (Fig. S4).

Loss of VTA NtsR1 Neurons Modifies the Mesolimbic DA System

Lastly, we examined how loss of VTA NtsR1 neurons impacts the integrity of the mesolimbic DA system. *NtsR1*^{DTA} mice have reduced TH and DAT immuo-labeled terminals in the NA and OFT compared to control mice (Fig. 6A, B) but residual immunoreactivity suggests preservation of non-NtsR1 expressing mesolimbic neurons. To assess molecular alterations in the VTA, we administered unilateral VTA injections of AAV-

DTA or AAV-GFP to *NtsR1*^{Cre}*;GFP* mice and analyzed the fold difference in gene expression between the injected and uninjected sides; we refer to these as *NtsR1*$^{GFP\text{-}Uni}$ and *NtsR1*$^{DTA\text{-}Uni}$ mice (Fig. 6C). As expected, *NtsR1* and DA-associated transcripts such as *Th*, *Dat* and *D2* were significantly reduced in the VTA of *NtsR1*$^{DTA\text{-}Uni}$ mice, consistent with the loss of DAergic VTA NtsR1 cell bodies (Fig. 6D). The slight reduction in *Vglut2* and *Vgat* in *NtsR1*$^{DTA\text{-}Uni}$ mice suggests that VTA NtsR1 neurons may also contain glutamate and/or GABA in addition to DA. *Ntsr2* expression, however, did not differ between *NtsR1*$^{DTA\text{-}Uni}$ mice and controls, consistent with our finding that NtsR1 and NtsR2 are expressed on distinct VTA cell populations (Fig. 1). As expected, the fold change in expression between injected and uninjected sides was close to 1.0 in the in *NtsR1*$^{GFP\text{-}Uni}$ controls, validating the use of the uninjected side as an internal comparison for each mouse. No differences in NA expression of *D1* or *D2* were observed between groups (Fig. 6E). In sum, loss of VTA NtsR1 neurons results in structural and molecular adaptations in the mesolimbic DA system but does not abrogate the entirety of DA-mediated signaling that is required for survival.

DISCUSSION

The lack of molecular markers to differentiate functionally heterogeneous and projection-specified VTA DA neurons has impeded understanding of how these neurons orchestrate behavior and body weight. We show that NtsR1 expression identifies a subset of VTA DA neurons that projects preferentially to the ventral striatum compared to the PFC, and thus represents a molecular marker of primarily mesolimbic, not mesocortical VTA DA neurons. Loss of this VTA NtsR1 population alters DA-mediated behaviors to protect against weight gain without compromising DA-mediated signaling necessary for survival (Zhou and Palmiter, 1995). Collectively, our data identify VTA NtsR1 neurons as important coordinators of energy intake and output behaviors that determine body weight.

Since Nts actions via NtsR1 and NtsR2 are implicated in control of distinct physiology (Geisler et al., 2006), we hypothesized that these receptors might identify functionally distinct DA neurons. We therefore used Cre-mediated reporter mice to identify the cells expressing each receptor isoform, confirming that NtsR1 is almost exclusively expressed by DA neurons while NtsR2 is primarily expressed by non-DAergic neurons and astrocytes in the VTA. Since NtsR1 is a $G\alpha_q$-coupled GPCR (Tanaka et al., 1990), our findings indicate a mechanism for Nts to directly enhance the activation of NtsR1-expressing DA neurons, consistent with the established roles for NtsR1 in promoting DA release to the striatum and DA-mediated locomotor activity (Remaury et al., 2002; Steinberg et al., 1994), anorexia (Remaury et al., 2002), and reward (Kempadoo et al., 2013; Rouibi et al., 2015) Our data do not, however, rule out roles for the non-DAergic populations of NtsR2 cells to indirectly promote DA signaling. The TH-negative NtsR2 neurons found primarily within the ventromedial, GABA-rich portion of the VTA may disinhibit local DA neurons and promote their activation (Kempadoo et al., 2013; Piccart et al., 2015). Nts may also act via the numerous NtsR2-expressing astrocytes within the VTA to indirectly modify DA-mediated behavior, consistent with reports of glial cells mediating NtsR2-dependent fear behavior (Yamauchi et al., 2007). Going forward, it will be important to define the neural circuits by

which Nts engages these distinct NtsR1 and NtsR2-expressing cell types and their individual contributions to DA-mediated behaviors and energy balance.

In situ hybridization predicts a larger population of NtsR1-expressing neurons in the VTA than were identified using transgenic NtsR1^{Cre}-GFP mice (Allen Brain Atlas - (Lein et al., 2007) and Fig. 1). This discrepancy may be due to low Cre expression common to transgenic lines, diminishing the intensity of Cre-mediated GFP expression necessary to identify NtsR1 cells. Increasing the concentration of Cre-inducible transcripts can enhance recombination, and indeed, injecting *NtsR1^{Cre}* mice with AAV-GFP identifies additional NtsR1 neurons in the VTA. The AAV-identified VTA NtsR1-GFP neurons exclusively co-label with a subpopulation of TH cells, confirming that VTA NtsR1 neurons are a subset of all DA neurons (Fig. S5). In sum, these data suggest that the VTA NtsR1 neuronal population is larger than depicted in Fig. 1, but that subsequent AAV manipulations were able to transduce and modulate most VTA NtsR1 neurons.

Although generalized depletion of VTA DA neurons does not alter locomotor activity or energy intake (Drui et al., 2014; Ouachikh et al., 2013; Pioli et al., 2008), ablation of the specific subset of VTA NtsR1 neurons altered both behaviors and prevented age-associated and diet-induced weight gain. Since aging and obesogenic environment are two significant instigators of human weight gain, our findings suggest roles for VTA NtsR1 neurons in countering the development of obesity. Intriguingly, the pronounced physical activity and energy expenditure of *NtsR1^{DTA}* mice appears to be the primary source of protection from weight gain. Increased physical activity is similarly impactful in humans, as individuals who exercise are more likely to avoid weight gain with age (Lee et al., 2010; Shiroma et al., 2012). Although the *NtsR1^{DTA}* mice eat slightly more than controls, their energy intake is only sufficient to compensate for elevated levels of activity and energy expenditure, and did not promote weight gain. Thus, mesolimbic VTA NtsR1 neurons are not essential drivers of feeding *per se*, but they coordinate energy intake required to support physical activity.

Given that VTA NtsR1 neurons project heavily to the ventral striatum, where DA release regulates the motivation for palatable foods (Day et al., 2007; McCullough et al., 1993), we were surprised that *NtsR1^{DTA}* mice did not differ from controls in their willingness to work for sucrose rewards. Furthermore, *NtsR1^{DTA}* mice demonstrated intact liking and wanting of palatable foods. These findings suggest that VTA NtsR1 neurons are not required for DA-mediated ingestive behavior, and that other non-NtsR1 expressing VTA neurons exert this control. It may be argued, however, that *NtsR1^{DTA}* mice with low body weight and adiposity should display increased motivation for sucrose (Fulton et al., 2000), and thus have a deficit in coordinating intake behavior to meet energy demands. This is consistent with our findings that *NtsR1^{DTA}* mice also lack the ability to adapt motivated intake in the face of satiety cues such as sucrose pre-feeding and leptin treatment (Sharma et al., 2012). Taken together, these data suggest that VTA NtsR1 neurons are not required for the execution of motivated food intake, but rather serve to tune it in response to peripheral energy needs.

VTA NtsR1 neurons must receive information about peripheral energy status to coordinate metabolic state and DA-mediated behavior. The adipocyte-derived hormone leptin modulates energy balance in part via the actions of Nts and NtsR1 (Kim et al., 2008; Opland

et al., 2013; Sahu et al., 2001), and we demonstrate an obligate role of VTA NtsR1 neurons in this process. It is unlikely that VTA NtsR1 neurons are direct targets of leptin because VTA NtsR1- and leptin receptor (LepRb) expressing populations have distinct anatomical distributions and projection sites (Fig. 2 and (Leshan et al., 2010)), suggesting that they comprise separate neuronal populations. Leptin does, however, activate LepRb neurons of the lateral hypothalamic area (LHA) that project to and release Nts into the VTA, promoting NtsR1-dependent release of DA in the NA (Opland et al., 2013; Patterson et al., 2015). LHA LepRb neurons may thus be the neural hubs linking leptin action with Nts and DA signaling.

We used NtsR1 as a marker to identify VTA NtsR1 neurons, but it remains to be determined which signals from these neurons are critical for regulation of energy balance. VTA NtsR1 neurons may co-express both GABA and/or glutamate to modify activation of striatal targets. Nts action via NtsR1 expressed on VTA NtsR1 neurons may also contribute to DA-mediated behaviors. Indeed, the phenotype of *NtsR1*^{DTA} mice resembles whole-body NtsR1 knockout mice with hyperactivity, increased sucrose preference, and lack of adaptive response to leptin (Opland et al., 2013). Together, these models argue that chronic disruption of Nts signaling via VTA NtsR1 neurons biases homeostasis toward energy expenditure. Perhaps the most important signal from VTA NtsR1 neurons may be DA itself. Although *NtsR1*^{DTA} mice exhibit substantial loss of VTA NtsR1 neurons and diminished DAergic projections to the NA, their intact AMPH-induced cFos confirms that at least some DA neurons remain and are functional. If anything, chronic loss of VTA NtsR1 neurons may lead to enhanced DA signaling. Indeed, mice lacking VTA NtsR1 neurons display increased general ambulatory behavior, but decreased AMPH-induced locomotor activity accompanied by stereotypy, a behavioral response typically associated with high-dose AMPH and ceiling-levels of DA efflux (Yates et al., 2007). We thus speculate that loss of VTA NtsR1 neurons leads to increased striatal DA signaling, and AMPH further increases DA to levels that promote stereotypy instead of augmenting ambulatory activity. Numerous mechanisms could enhance DA action, such as altered balance of D1/D2 protein expression or impaired DAT kinetics, and will be important to define in the future.

Our data indicate that loss of VTA NtsR1 neurons prevents diet-induced obesity in mice, hence VTA NtsR1 neurons may represent a cellular target for preventing and treating human obesity. Ablation of neurons is not a feasible therapeutic strategy, thus it will be imperative to identify pharmacological means to selectively modulate VTA NtsR1 neurons to promote behaviors that prevent weight gain. Previous work demonstrates that pharmacologic treatment of Nts or Nts agonists in the VTA suppresses motivated feeding (Kelley et al., 1989) and enhances locomotor activity in an NtsR1-dependent manner (Elliott and Nemeroff, 1986; Steinberg et al., 1994). Conversely, other studies showed suppression of locomotor activity when Nts was applied directly to the NAc (Kalivas et al., 1984), underscoring the need for circuit-specific interventions. Although developmental loss of NtsR1 promotes palatable food intake and weight gain (Opland et al., 2013), our current data show that selective ablation of VTA NtsR1 neurons in adult mice protects them from obesity, suggesting that pharmacological modulation of Nts signaling may be useful in adults with established VTA NtsR1 DA circuits. Going forward, it will be important to define how Nts and other signals engage VTA NtsR1 neurons to identify strategies that prevent obesity

EXPERIMENTAL PROCEDURES

Mice

NtsR1^{Cre} mice were purchased from the Mutant Mouse Regional Resource Center, UC Davis (B6.FVB(Cg)-Tg(NtsR1-cre)GN220Gsat/Mmucd, Stcok number 030648-UCD). *NtsR2*^{Cre} mice were generated via homologous recombination (knock-in) methods described previously (Leinninger et al., 2011). To visualize NtsR1 and NtsR2-expressing neurons, *NtsR1*^{Cre} and *NtsR2*^{Cre} mice were bred to a Cre-inducible Rosa$^{eGFP\text{-}L10a}$ reporter line (Krashes et al., 2014), generating mice that express GFP selectively in NtsR1 and NtsR2 neurons (*NtsR1*^{Cre}*;GFP* and *NtsR2*^{Cre}*;GFP*); brains from adult male mice were analyzed for GFP. To induce Cre expression in *NtsR2*^{Cre}*;GFP* mice, the *frt*-flanked Neo cassette was removed by injecting FlpO adenovirus (Vector Biolabs) into the lateral ventricles of adult animals (see Supp. Methods). Mice were bred and housed in a 12h light/12h dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines.

To generate *NtsR1*^{GFP} and *NtsR1*^{DTA} study mice, 8–10 wk old *NtsR1*^{Cre}*;GFP* males received bilateral injections of either 150nL Cre-inducible AAV-GFP(rAAV2/hSvn-DIO-eGFP, University of North Carolina Vector Core) or AAV-DTA (lox-mCherry-loxDTA-WPRE-AAV, serotype 10), which expresses the cytotoxic Subunit A of Diptheria Toxin in the presence of Cre into the VTA (A/P: −3.2, M/L: +/−0.48, D/V: −4.65). Only mice found to have injections contained within the VTA were included in the final analysis.

Metabolic Profiling

At 4, 8 and 16 wk post-surgery the mice were analyzed for body composition using an NMR-based instrument (Minispec mq7.5, Bruker Optics). At 4 and 16 wk post-surgery, mice were placed in TSE cages for metabolic phenotyping (PhenoMaster, TSE Systems). After 24 hours of acclimation, mice were continuously monitored for food and water intake, locomotor activity, and energy expenditure over 4 days. Ambient temperature was maintained at 20–23°C and the airflow rate through the chambers was adjusted to maintain an oxygen differential around 0.3% at resting conditions. Metabolic parameters including VO_2, respiratory exchange ratio, and energy expenditure were assessed via indirect calorimetry by comparing O_2 and CO_2 concentrations relative to a reference cage.

Operant Testing

Standard Testing: NtsR1^{DTA} and *NtsR1*^{GFP} were trained to nose-poke for unflavored 20 mg sucrose pellets (TestDiet 1811555) using operant-responding chambers (Med Associates) as previously described (Sharma et al., 2012). See Supp. Methods for more detail. *Sucrose pre-feeding:* After achieving stable PR, mice were given *ad lib* access to 3g of sucrose pellets in their home cages overnight. The following morning they were re-tested on the PR schedule. *Leptin Treatment:* Mice with stable PR responses were injected with PBS or leptin (5mg/kg, ip) on different days and tested one hour later.

Open Field Locomotor Activity

Open field locomotor activity was assessed using a digital CCD camera and video-tracking software (Clever Sys) (Eagle et al., 2015). Mice were tested on 3 separate days; each day, mice were placed in the boxes to acclimate, followed by an *ip* injection of PBS 30 min later. At 60 min, mice received either a second injection of PBS, or SCH23390 (0.1mg/kg) or amphetamine (4mg/kg) and recorded for another 60 minutes.

Statistics

Student's t-tests and 2-way ANOVA were calculated using Prism 6 (GraphPad). For all metabolic data, analysis of covariance (ANCOVA) was computed in SPSS 22 (IBM). Body weight was analyzed as a covariate to correct for any inherent differences it may have on metabolism (Tschop et al., 2012). All data were tested for homogeneity of regression, independence of the covariate (body weight), and linearity of regression prior to running the ANCOVA. For all data, *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- NtsR1 is expressed on a subset of VTA DA neurons that projects to the NA
- Loss of VTA NtsR1 neurons promotes energy use and prevents diet-induced obesity
- VTA NtsR1 neurons are necessary to coordinate energy cues with ingestive behavior
- Ablation of VTA NtsR1 neurons alters expression of mesolimbic DA markers

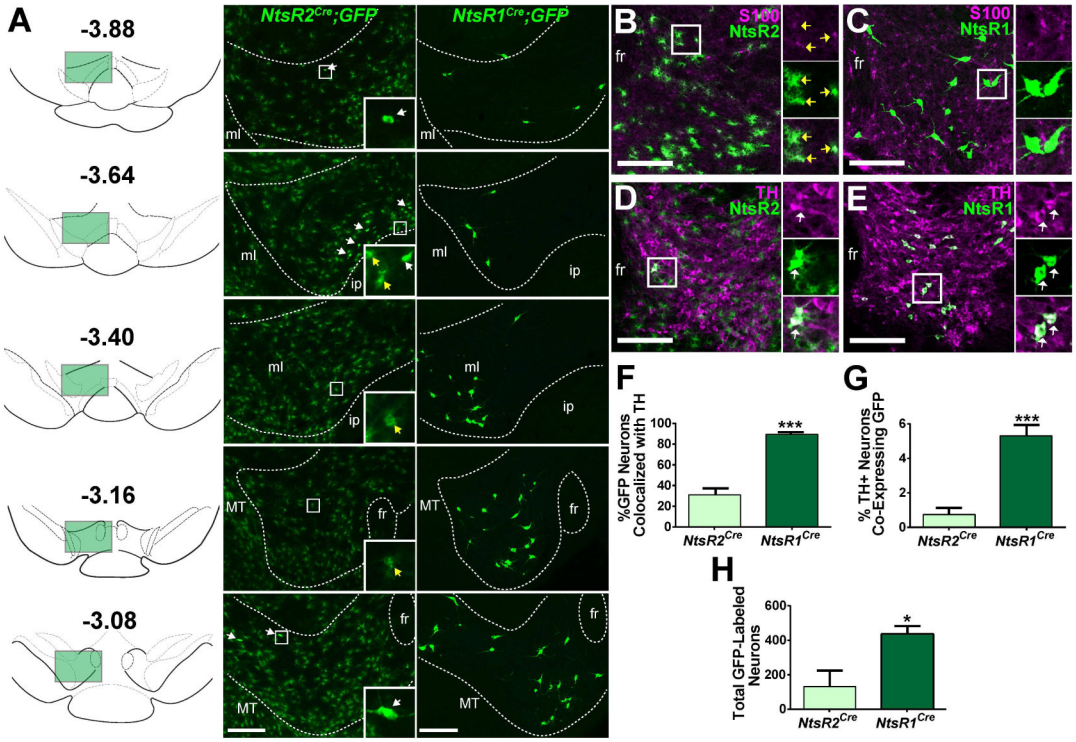

Figure 1. Distribution and neurochemical phenotype of VTA NtsR1 and NtsR2 neurons

VTA NtsR1 and NtsR2 neurons were visualized by crossing *NtsR1*^{Cre} and *NtsR2*^{Cre} mice to a Cre-inducible GFP reporter line. *See* Fig. S1 and S2A *for more detail*. **A)** Rostrocaudal distribution of VTA NtsR1 and NtsR2 neurons with bregma positions per (Paxinos and Franklin, 2001). White arrows denote neurons, yellow arrows denote glial cells. **B)** Colocalization of glial cell marker S100 (purple) in the VTA with NtsR2 cells (green) and **C)** NtsR1 cells (green). **D)** Colocalization of tyrosine hydroxylase (TH, purple) in the VTA with NtsR2 cells (green) and **E)** NtsR1 cells (green). **F)** Percentage of NtsR1 and NtsR2 neurons that colocalize with TH. **G)** Percentage of TH positive neurons that colocalize with NtsR1 or NtsR2. **H)** Total number of GFP-identified NtsR1 and NtsR2 neurons in the VTA. *NtsR1*^{Cre}*;GFP* n=4, *NtsR2*^{Cre}*;GFP* n=6. Data represent mean ± SEM, *p < 0.05, ***p < 0.001 determined by unpaired t-test. Scale bars = 100 μm. Abbreviations: ml = medial lemniscus; ip = interpeduncular nucleus; fr = fasciculus retroflexus; MT = medial terminal nucleus of the accessory optic tract.

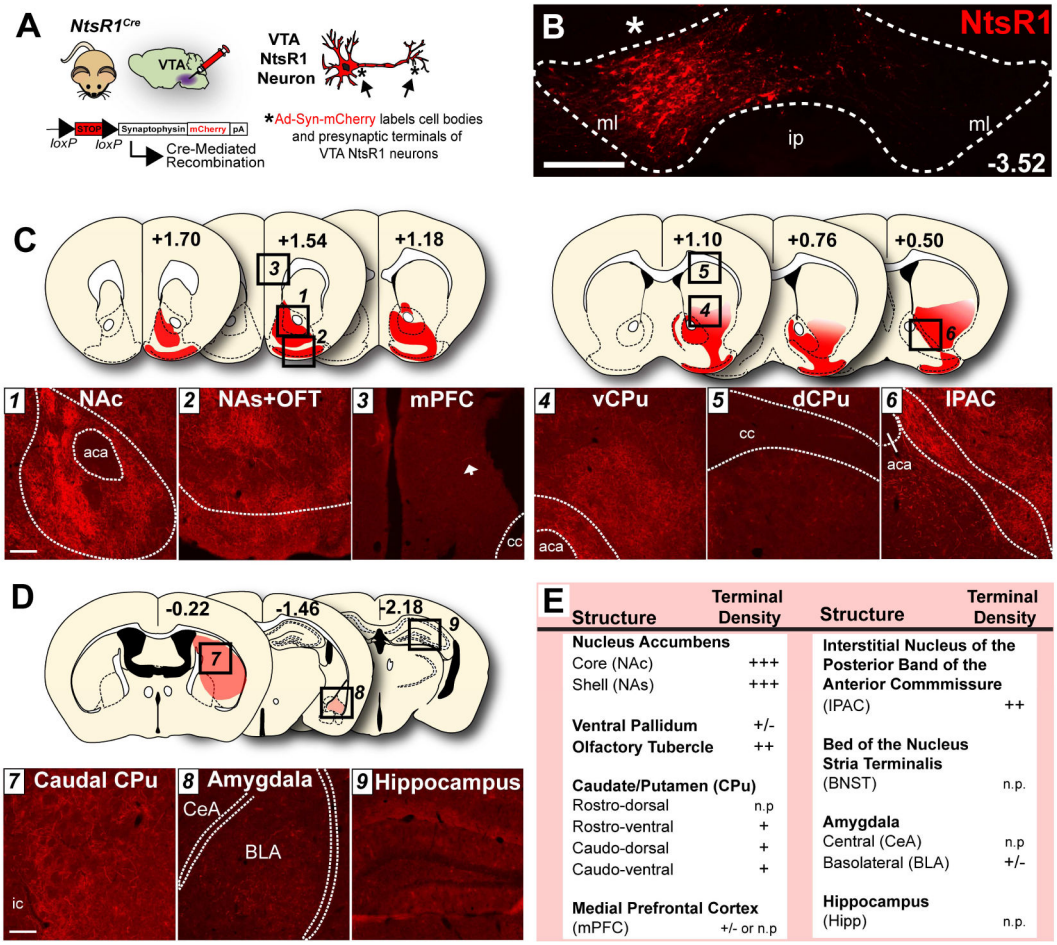

Figure 2. VTA NtsR1 neurons project to the ventral striatum

A) *NtsR1*^{Cre} mice were unilaterally injected in the VTA with Ad-Syn-mCherry, resulting in syn-mCherry expression in the cell bodies and presynaptic terminals of VTA NtsR1 neurons (n=5). **B)** VTA injection site (asterisk) with mCherry-labeled NtsR1 cell bodies. Scale bar = 200 μm. **C, D)** Efferent targets of VTA NtsR1 neurons. Numbered dashed-boxes in coronal schematic images correspond to microscopy images below. Bregma positions are according to (Paxinos and Franklin, 2001). Scale bars in **C, D** = 100 μm. **E)** Table summarizing relative NtsR1 terminal density across multiple brain areas with emphasis on previously established VTA efferents. KEY: +++ heavy; ++ moderate; +light, +/-; sparse; n.p.= not present. Abbreviations: aca=anterior commissure, cc=corpus callosum, ic=internal capsule, ml=medial lemniscus, ip=interpeduncular nucleus, CeA= central amygdala, BLA= basolateral amygdala.

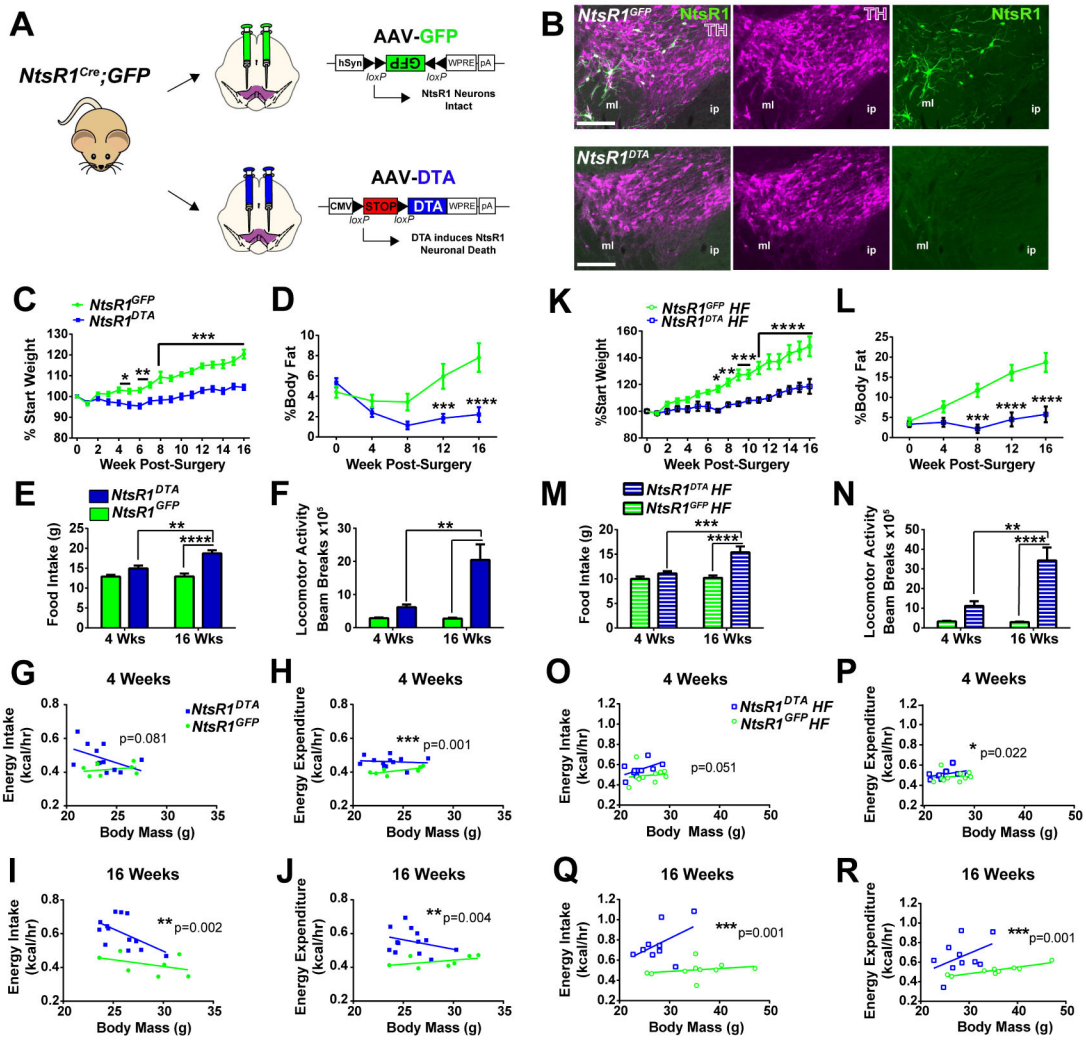

Figure 3. Loss of VTA NtsR1 neurons disrupts energy balance

A) 8 wk old male *NtsR1^{Cre};GFP* mice received bilateral injections of AAV-DTA or AAV-GFP in the VTA. **B)** Images show representative NtsR1-GFP (green) and TH (purple) expression from *NtsR1^{GFP}* and *NtsR1^{DTA}* mice 2 weeks after surgery. Scale bars =200μm. Abbreviations: ml=medial lemniscus, ip=interpeduncular nucleus. **C–J)** Metabolic assessment of *NtsR1^{GFP}* and *NtsR1^{DTA}* mice on chow diet over 4 days (3.1 kcal/g) (*NtsR1^{GFP}* n=7, and *NtsR1^{DTA}* n=13). **C)** Percentage change in body weight from starting weight and **D)** percentage of body fat determined via NMR spectroscopy. **E)** Chow intake and **F)** total locomotor activity as measured in TSE metabolic cages. **G)** Weight-adjusted energy intake and **H)** energy expenditure at 4 wk post surgery and **I)** weight-adjusted energy intake and **J)** energy expenditure measured at 16 wk post surgery. **K–R)** Metabolic assessment of *NtsR1^{GFP}* and *NtsR1^{DTA}* mice on HF diet (4.7 kcal/g) (*NtsR1^{GFP} HF* n=10 and *NtsR1^{DTA} HF* n=10) over 4 days. **K)** Percentage change in body weight from starting weight, **L)** percentage of body fat, **M)** HF diet intake and **N)** total locomotor activity. **O)** Energy intake and **P)** energy expenditure at 4 wk post surgery, and **Q)** energy intake and **R)** energy expenditure at 16 wk post surgery, normalized to body mass. For **C–F** and **K–N**,

graphed data represent mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001 analyzed by two-way ANOVA with Bonferroni post-tests. For scatterplots in **G–J and O–R**, data were analyzed using ANCOVA to account for body weight as a covariate and p values are indicated on graphs. *See* Fig. S3 *for additional metabolic data.*

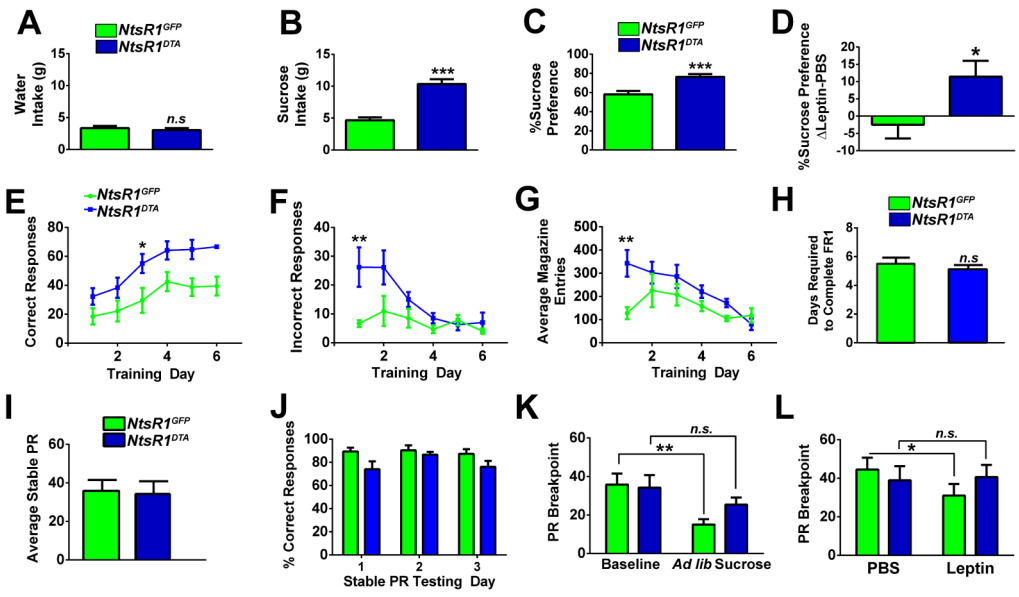

Figure 4. VTA NtsR1 neurons modulate hedonic and motivated ingestive behavior

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

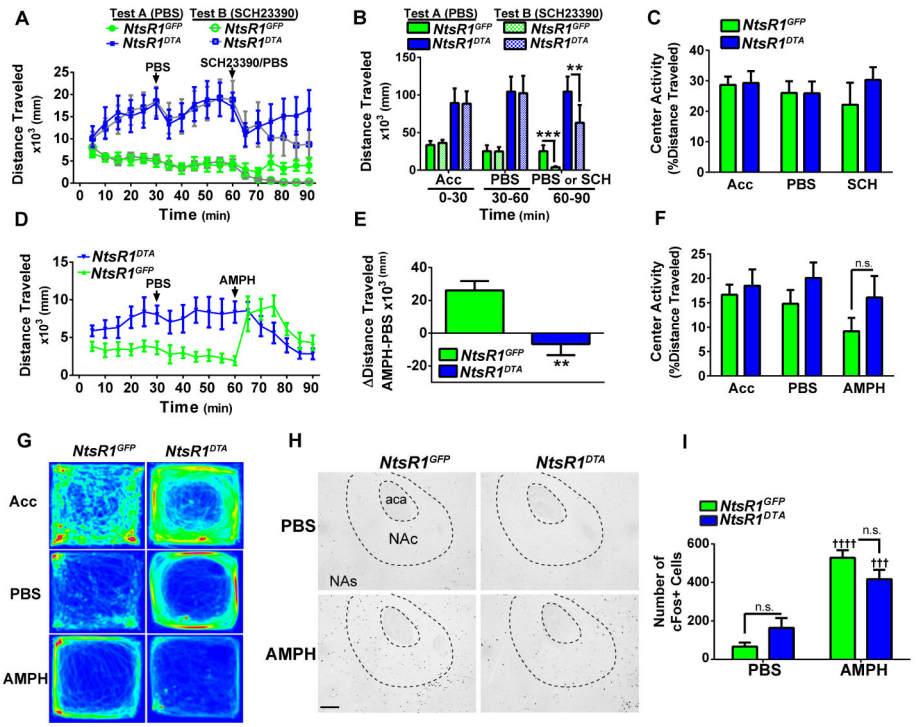

Figure 5. Loss of VTA NtsR1 neurons alters response to amphetamine

A) Locomotor activity of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice in open field boxes during acclimation, treatment with PBS, and either PBS (Test A) or D1R antagonist SCH23390 (0.1 mg/kg, ip) (Test B). Data represent average distance traveled per 5 min interval ± SEM (*NtsR1*^{GFP} n=6, and *NtsR1*^{DTA} n=10). **B)** Mean distance traveled during treatment period ± SEM, analyzed by two-way ANOVA. **C)** Percentage of distance traveled in the center zone of boxes over each testing period. **D)** Open field locomotor activity of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice after treatment with PBS and AMPH (4 mg/kg, ip). Data represent average distance traveled per 5 min interval ± SEM (*NtsR1*^{GFP} n=6, and *NtsR1*^{DTA} n=10). **E)** Mean difference between locomotor activity after AMPH treatment relative to PBS ± SEM, **p=0.003. **F)** Percentage of distance traveled in the center zone over each period ± SEM, n.s. = not significant. *See* Fig. S4 *for additional anxiety data.* **G)** Representative heat maps demonstrating mouse location during acclimation (Acc), PBS, and AMPH treatments. **H)** cFos expression in the NA (+1.34 bregma) of *NtsR1*^{GFP} and *NtsR1*^{DTA} treated with PBS or AMPH 90 minutes prior to perfusion. Abbreviations: aca = anterior commissure; NAc = nucleus accumbens core; NAs = nucleus accumbens shell. **I)** Number of cFos positive (cFos +) cells in NA. Graphed data represent mean ± SEM, analyzed by two-way ANOVA. ǂ ǂ ǂ ǂ p<0.0001, ǂ ǂ ǂ p<0.001, indicates significant difference between PBS and amphetamine treatment within each group, (*NtsR1*^{GFP} PBS n=3, AMPH=3; *NtsR1*^{DTA} PBS n=3, AMPH n=5).

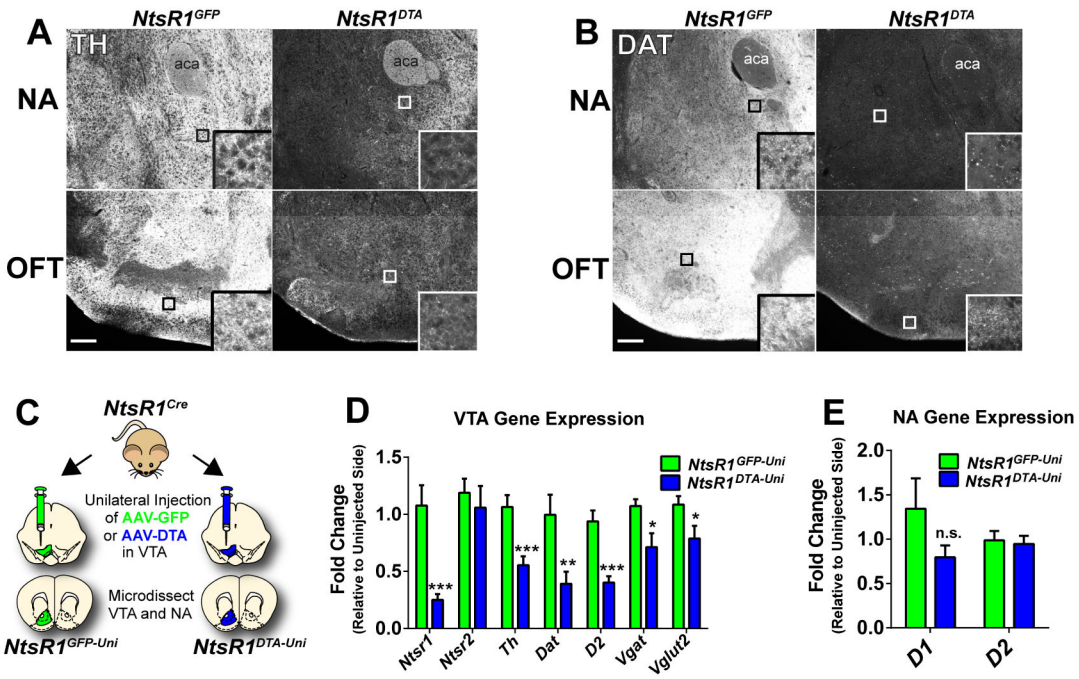

Figure 6. Ablation of VTA NtsR1 neurons decreases markers of DA signaling

Post-hoc analysis of **A)** tyrosine hydroxylase (TH) and **B)** dopamine transporter (DAT) immunoreactivity in the NA and OFT (+1.50 bregma) of *NtsR1*^{GFP} and *NtsR1*^{DTA} mice perfused 10–13 months after surgery. Scale bars = 100 μm. Abbreviation: aca = anterior commissure. White boxes identify digitally enlarged areas from each image. **C)** *NtsR1*^{Cre} mice were injected unilaterally with AAV-DTA to ablate VTA NtsR1 neurons or AAV-GFP as a control. The VTA and NA were dissected separately from each side of the brain and expression of DA-related genes was assessed at 16 wks post-surgery in the **D)** VTA and **E)** NA. Fold change was determined for each animal relative to the uninjected side. Data are expressed as mean ± SEM (*NtsR1*^{GFP} n=6–7, *NtsR1*^{DTA} n=8–9). Significant differences were determined by unpaired t-test *p < 0.05, **p < 0.01, ***p < 0.001.