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Neuroendocrine Signals Act In The Hindbrain And Mesopontine Tegmentum To Control Energy Balance

David J. Reiner
University of Pennsylvania, david.reiner811@gmail.com

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Abstract
With the high prevalence of overweight and obese people in the United States, understanding the endogenous systems that control energy balance is of clinical significance. Given that obesity is driven, in part, by excessive intake of calories, it is critical to understand how food intake is controlled, with the goal of improving pharmacotherapies to treat obesity. A variety of peripherally and centrally-derived neuroendocrine signals are released following ingestion and act in distributed nuclei throughout the brain to control energy balance. The contribution of particular nuclei and the specific cell types within those nuclei that impact energy balance control requires further investigation. This dissertation focuses on novel mechanisms of two anorexogenic hormones, glucagon-like peptide-1 (GLP-1) and amylin, for energy balance control. Given that glutamatergic signaling mediates the intake suppressive effects of GLP-1 and that astrocytes are key modulators of synaptic glutamate levels, I hypothesize that GLP-1 receptors (GLP-1R) are expressed on astrocytes within the nucleus tractus solitarius (NTS), a feeding relevant nucleus, and contribute to energy balance control. Furthermore, neurons within the NTS produce GLP-1 and project widely throughout the brain. Given that the lateral dorsal tegmental nucleus (LDTg), an understudied region in energy balance control, receives projections from the NTS and expresses the GLP-1R, I hypothesize that GLP-1R signaling in the LDTg regulates energy balance control. Traditionally believed to act in the area postrema, the hormone amylin has recently been shown to act in mesolimbic regions to control energy balance, findings that have broadened the perspectives of CNS amylin action. Given that the LDTg binds amylin and receives and send projections to mesolimbic sites, I hypothesized that the amylin receptor signaling in the LDTg modulates energy balance. Collectively, the data presented in this dissertation broaden our understanding of the central nervous system (CNS) action of neuroendocrine signals for energy balance control, in terms of both cell- and nuclei-specificity, providing greater insight into potential targets for the developing improved pharmacotherapies to treat obesity.

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Supervisor of Dissertation

_____

Matthew R. Hayes, Ph.D.

Associate Professor of Nutritional Neuroscience in Psychiatry

Graduate Group Chairperson

_____

Joshua I. Gold, Ph.D.

Professor of Neuroscience

Dissertation Committee

Heath Schmidt, Ph.D., Assistant Professor of Psychiatry (Chair)

J. Nicholas Betley, Ph.D., Assistant Professor of Biology

Irwin Lucki, Ph.D., Emeritus Professor of Psychology in Psychiatry

Kendra Bence, Ph.D., Senior Director of Metabolism, Pfizer (External Member)

To my grandparents, Magdalena and John Reiner and Loretta and Jerome Honig.

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ABSTRACT

NEUROENDOCRINE SIGNALS ACT IN THE HINDBRAIN AND MESOPONTINE TEGMENTUM TO CONTROL ENERGY BALANCE

David Joseph Reiner

Matthew R. Hayes, Ph.D.

With the high prevalence of overweight and obese people in the United States, understanding the endogenous systems that control energy balance is of clinical significance. Given that obesity is driven, in part, by excessive intake of calories, it is critical to understand how food intake is controlled, with the goal of improving pharmacotherapies to treat obesity. A variety of peripherally and centrally-derived neuroendocrine signals are released following ingestion and act in distributed nuclei throughout the brain to control energy balance. The contribution of particular nuclei and the specific cell types within those nuclei that impact energy balance control requires further investigation. This dissertation focuses on novel mechanisms of two anorexogenic hormones, glucagon-like peptide-1 (GLP-1) and amylin, for energy balance control. Given that glutamatergic signaling mediates the intake suppressive effects of GLP-1 and that astrocytes are key modulators of synaptic glutamate levels, I hypothesize that GLP-1 receptors (GLP-1R) are expressed on astrocytes within the nucleus tractus solitarius (NTS), a feeding relevant nucleus, and contribute to energy balance control. Furthermore, neurons within the NTS produce GLP-1 and project widely throughout the brain. Given that the lateral dorsal tegmental nucleus (LDTg), an understudied region in energy balance control, receives projections from the NTS and expresses the GLP-1R, I hypothesize that GLP-1R signaling in the LDTg regulates

energy balance control. Traditionally believed to act in the area postrema, the hormone amylin has recently been shown to act in mesolimbic regions to control energy balance, findings that have broadened the perspectives of CNS amylin action. Given that the LDTg binds amylin and receives and send projections to mesolimbic sites, I hypothesized that the amylin receptor signaling in the LDTg modulates energy balance. Collectively, the data presented in this dissertation broaden our understanding of the central nervous system (CNS) action of neuroendocrine signals for energy balance control, in terms of both cell- and nuclei-specificity, providing greater insight into potential targets for the developing improved pharmacotherapies to treat obesity.

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CHAPTER 1: INTRODUCTION

The prevalence of obesity in the United States has increased over the past several decades, with currently 36.5% of adults classified as obese by Body Mass Index (BMI) (Ogden et al., 2015). Obesity is characterized by a chronic state of positive energy balance and weight gain, driven in part by caloric intake beyond metabolic need (Narayanan et al., 2010; Kenny, 2011; Grill and Hayes, 2012). This hyperphagia typically occurs in times of energy abundance, largely because of non-homeostatic feeding of highly caloric and rewarding palatable foods (Lutter and Nestler, 2009; Halford et al., 2010). However, the causes of and therapeutic treatment strategies to prevent this chronic hyperphagia remain unclear. As obesity is co-morbid with heart disease, stroke, diabetes, and cancer, each obese patient pays on average an additional $1,429 annually compared to a normal weight age-matched patient (Finkelstein et al., 2009). In addition, over $147 billion in annual direct medical care is spent on obese patients, placing an extreme burden on the US health care system (Finkelstein et al., 2009). It is therefore important to develop effective treatments for obesity. However, individuals attempting to lose weight by diet and exercise often fail, and bariatric surgery is invasive and expensive. Pharmacotherapy may be a more effective approach to treat obesity, but current non-invasive therapeutic pharmacological agents produce only a ~5-10% reduction in weight loss and are limited by adverse side effects and restricted use with certain co-morbidities (Cooke and Bloom, 2006; Dunican et al., 2010; Gadde and Pritham Raj, 2017). By examining the endogenous systems that govern energy balance, novel and specific therapeutic targets can be identified for obesity treatment.

Energy balance in mammals is controlled by a complex set of neuroendocrine signals that act together to regulate energy intake and expenditure (Grill and Hayes,

2012). These include neural signals, such as gut-brain communication via the vagus nerve, as well as numerous hormones produced peripherally and centrally. Many of these hormones, such as glucagon-like peptide-1 (GLP-1) (Hayes et al., 2010; Hayes, 2012; Hayes et al., 2014a) and amylin (Mack et al., 2007; Mack et al., 2010; Mietlicki-Baase et al., 2013b), affect food intake and the incentive value of food by binding to their individual receptors on vagal afferents or directly in the central nervous system (CNS), ultimately leading to alterations in physiology and behavior (Berthoud, 2002; Grill and Kaplan, 2002; Lutz, 2006; Grill and Hayes, 2012; Hayes et al., 2014a; Mietlicki-Baase and Hayes, 2014; Hayes and Schmidt, 2016; Kanoski et al., 2016; Mietlicki-Baase, 2016). While these signals are processed by a distributed set of CNS structures, the contribution of particular nuclei and the specific cell types within those nuclei that impact energy balance control still remain largely unknown. It is therefore crucial to understand the CNS circuitry engaged by these energy status signals to modulate energy balance and the rewarding value of food, as this may enable us to develop improved therapeutic targets for obesity.

This dissertation focuses on examining novel mechanisms governing energy balance control. Experiments focus on the role of astrocytes, an often-overlooked regulator of synaptic neurotransmission, in energy balance control. Cell-type-specific effects (e.g. neurons vs. astrocytes) of GLP-1 receptor (GLP-1R) signaling are investigated in the context of mediating energy balance control. Further studies analyze the individual roles of GLP-1 and amylin for energy balance control in an understudied brain region for motivated behaviors, the lateral dorsal tegmental nucleus (LDTg).

Astrocytes: A general overview

"What functional significance can be attributed to the neuroglia? Unfortunately, the present state of science does not allow to answer the important question but through more or less rational conjectures. When facing this problem, the physiologist is totally disarmed for lack of methods." Santiago Ramon y Cajal, 1899, Textura del Sistema Nervioso del Hombre y de los Vertebrados.

First termed neuroglia, glia were first described by Rudolf Virchow in 1846 as the "connective tissue" or "cement" of the brain [see (Garcia-Marin et al., 2007) for review]. However, in comparison to neurons, glia have historically been neglected in neuroscience as glia are not electrically excitable. Currently, while the number of research articles on the role of neurons is still extremely high compared to glia, we know that there are nearly as many neurons as glial cells in the brain and over eight types of glia have been identified, including astrocytes (Garcia-Marin et al., 2007; Herculano-Houzel, 2014; von Bartheld et al., 2016). Named for their star-like shape, astrocytes were long thought of as the "glue" of the brain and silent partners in brain function and plasticity (Garcia-Marin et al., 2007). Over one hundred years ago and largely based off evidence from static images of astrocyte staining, Santiago Ramon y Cajal proposed physiological roles for astrocytes in brain function as well as physiology and behavior, including the potential involvement of astrocytes in attention and sleep/wake states [see (Garcia-Marin et al., 2007; Navarrete and Araque, 2014) for review]. With the emergence of advanced techniques to study astrocytes, these proposals have largely been confirmed in recent years. Indeed, findings have illuminated the diverse roles astrocytes play in synaptic activity, forming what is termed a tripartite synapse, consisting of a presynaptic and a postsynaptic neuron and an astrocyte (Nedergaard et al., 2003; Ransom et al., 2003; Barres, 2008; Eroglu and Barres, 2010; Clarke and Barres, 2013;

Robinson and Jackson, 2016). Astrocytes have a critical role in regulating synaptic activity and express receptors for a variety of neurotransmitters. Upon neurochemical stimulation of astrocytes, intracellular calcium levels increase and astrocytes can release gliotransmitters (Nedergaard et al., 2003; Ransom et al., 2003; Halassa et al., 2007; Barres, 2008; Eroglu and Barres, 2010; Clarke and Barres, 2013; Robinson and Jackson, 2016). Astrocytes are also key modulators of synaptic and extrasynaptic glutamate levels through expression of glutamate transporters such as the glutamate transporter-1 (GLT-1) and the glutamate-aspartate transporter (GLAST) [see (Perego et al., 2000; Rimmele and Rosenberg, 2016) for review]. Recently, these cellular and synaptic functions of astrocytes have been shown to be relevant for physiology and behavior (Florian et al., 2011; Bull et al., 2014; Fields et al., 2014; Scofield and Kalivas, 2014; Scofield et al., 2015), including an understudied but potentially important role in energy balance control (Yi et al., 2011; Chowen et al., 2016; Leloup et al., 2016; Argente-Arizon et al., 2017).

Astrocytes: Roles in energy balance

Research has established that astrocytes sense a variety of nutrients and hormones from the blood and play dynamic roles in cellular energy homeostasis and energy balance control more broadly (Yi et al., 2011; Chowen et al., 2016; Leloup et al., 2016; Argente-Arizon et al., 2017). One of the first discoveries was that hypothalamic astrocytes express glucose transporters 1 and 2 (GLUT1 and GLUT2) and thereby have an important function in glycemic regulation, including sensing blood glucose, supplying glucose to nearby neurons, and ultimately controlling food intake [see (Leloup et al., 2016) for review].

Astrocytes contribute to synaptic and micro-circuit level plasticity in the development and maintenance of obesity and respond to neuropeptides [see (Dorfman and Thaler, 2015; Chowen et al., 2016) for review]. Obesity can be modeled by exposing animals to a high fat diet, which leads to hyperphagia, peripheral and central inflammatory responses and weight gain [see (Madsen et al., 2010; Dorfman and Thaler, 2015; Chowen et al., 2016; Guillemot-Legris and Muccioli, 2017) for review]. In the brain, increases in astrocyte number and changes in morphology, termed astrogliosis or gliosis, are hallmarks of inflammation and are caused by high fat diet exposure (Chowen et al., 2016). Changes in the number or morphology of astrocytes can lead to changes in cellular communication, nutrient and hormonal sensing from the blood, and release of cytokines associated with inflammation and oxidative stress (Chowen et al., 2016; Argente-Arizon et al., 2017). These changes may be protective following acute ingestion of a high fat diet, but chronic consumption of a high fat diet leads to maladaptive alterations in astrocyte expression and morphology, thereby perpetuating the overweight or obese state (Chowen et al., 2016; Argente-Arizon et al., 2017). However, the contribution of astrocytes to energy balance control has been examined almost exclusively in the hypothalamus, disregarding the potential contribution of astrocytes in other distributed brain regions relevant to energy balance control (Guillemot-Legris and Muccioli, 2017). Indeed, the role of numerous energy balance relevant hormones and neurotransmitters for astrocyte-mediated energy balance control requires further investigation. It is therefore crucial to understand, both in the “normal” lean state and in the obese state, the specific hormones or neuropeptides to which astrocytes respond, how astrocytes interact with neurons to control energy balance, and to identify areas of the brain in which astrocytes contribute to energy balance control.

The nucleus tractus solitarius (NTS) of the caudal brainstem

The importance of the caudal brainstem for feeding and energy balance control is highlighted by experiments done by Grill and colleagues dating back to the 1970s in which consummatory feeding was examined in chronic decerebrate rats, whose hindbrain and forebrain have been surgically separated (Grill and Norgren, 1978c, b, a; Flynn and Grill, 1988; Grill and Smith, 1988; Mark et al., 1988; Grill and Kaplan, 2001). Collectively, these studies showed that chronic decerebrate rats respond appropriately and similarly to intact rats in taste reactivity and single meal tests, suggesting that the caudal brainstem is sufficient for controlling ingestive behavior during short-term testing (Grill and Norgren, 1978c, b, a; Flynn and Grill, 1988; Grill and Smith, 1988; Mark et al., 1988; Grill and Kaplan, 2001). However, chronic decerebrate rats cannot engage in appetitive behaviors aimed at food procurement and do not appropriately compensate in meal size following food deprivation, indicating that the brainstem in isolation from the forebrain is not sufficient for the modulation of ingestive behavior over the long-term (Grill and Norgren, 1978c, b, a; Flynn and Grill, 1988; Grill and Smith, 1988; Mark et al., 1988; Grill and Kaplan, 2001). Together, these data underscore the importance of the hindbrain in integrating taste and gastrointestinal signals for acute control of meal size, but also emphasize the importance of hindbrain-forebrain communication in responding to the neuroendocrine signals that control the motivational aspects of feeding and long-term food intake (Grill and Kaplan, 2001).

The nucleus tractus solitarius (NTS) of the caudal brainstem is a critical nucleus in the regulation of energy balance. The NTS receives vagally-mediated gastrointestinal (GI) satiation signals and blood-borne energy status signals (Grill and Hayes, 2009, 2012). During ingestion, the GI tract senses chemical and nutritive properties of the

ingested food, stimulating the release of a number of gut peptides and neurotransmitters that can be released into the bloodstream or can bind to receptors on vagal sensory afferents, which first synapse in the NTS (Grill and Hayes, 2009, 2012). In addition, the NTS expresses receptors for a wide variety of peripherally and centrally-derived peptides and neurotransmitters (Grill and Hayes, 2009, 2012). The NTS therefore integrates diverse sensory information from the GI tract with circulating and centrally-derived neuroendocrine signals, and sends projections to other distributed brain areas to control feeding behavior and other aspects of energy balance control (Grill and Hayes, 2009, 2012).

The glucagon-like peptide-1 system in the NTS

Within the NTS, the GLP-1 system stands out for its biological and physiological relevance for energy balance control, as well as its clinical relevance for diabetes and obesity treatment. GLP-1 is an anorectic hormone released peripherally from intestinal L cells following meal initiation and centrally from preproglucagon (PPG) neurons in the NTS following a variety of stimuli, including gastric distension (Vrang et al., 2003; Grill and Hayes, 2012; Hayes et al., 2014a). Given that GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV), peripheral GLP-1 is thought to act primarily in a paracrine fashion on adjacent vagal afferents. In contrast, central GLP-1 is thought to act primarily as a neuropeptide, as PPG neurons project locally within the NTS and widely throughout the CNS (Rinaman, 2010; Dossat et al., 2011; Alhadeff et al., 2012; Kanoski et al., 2016). Therefore, centrally-produced GLP-1 is thought to be the main source of activation for GLP-1 receptor (GLP-1R) populations throughout the CNS, either through direct projections of PPG neurons or through volume transmission into the

cerebral spinal fluid (CSF) (Dossat et al., 2011; Alhadeff et al., 2012; Hsu et al., 2015; Kanoski et al., 2016).

PPG neurons in the NTS project widely throughout the brain, including but not limited to, mesolimbic regions such as the ventral tegmental area (VTA) and nucleus accumbens (NAc), as well as hypothalamic structures (Vrang et al., 2007; Dossat et al., 2011; Alhadeff et al., 2012; Dossat et al., 2013; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014; Burmeister et al., 2017). As PPG neurons are also glutamatergic (Zheng et al., 2014), it is believed that activation of PPG neurons (e.g. after gastric distension) would result in release of GLP-1 and/or glutamate release in downstream projection targets (either within the NTS or other CNS regions), though the precise dynamics of this release are not known.

Glucagon-like peptide-1 receptor signaling in the NTS

Within the NTS, GLP-1R signaling has been shown to modulate energy balance control. Direct NTS microinjection of the long-acting GLP-1R agonist exendin-4 (Ex-4) decreases food intake (Hayes et al., 2011b). In addition, blockade of NTS GLP-1Rs via microinjection of the competitive GLP-1R antagonist exendin-(9-39) (Ex-9) increases feeding and attenuates the intake suppression following gastric distension (Hayes et al., 2009). NTS GLP-1R activation also has a number of other feeding/energy balance-relevant effects, such as decreased intake of a palatable high fat diet, reduced conditioned place preference (CPP) for a palatable food, and lower progressive ratio responding for sucrose (a measure of motivation) (Alhadeff and Grill, 2014; Richard et al., 2015). Chronic knockdown of NTS GLP-1R results in increased chow intake and meal size as well as increased operant responding for sucrose on fixed ratio and

progressive ratio schedules of reinforcement (Alhadeff et al., 2016). Collectively, these data establish important pharmacological and physiological roles for NTS GLP-1R signaling in food intake control and motivated behavior. However, the specific cell types that bind GLP-1 and clinically relevant long-acting GLP-1R agonists within the NTS and the CNS in general have yet to be systemically evaluated.

GLP-1R activation decreases food intake at least in part through an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptor dependent process, suggesting that glutamate signaling following GLP-1R activation plays an important role in food intake suppression (Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014). GLP-1Rs are G-protein coupled receptors (GPCRs) coupled to G_s, and activation of GLP-1R leads to increased intracellular calcium and cAMP, PKA and MAPK activation, and downregulation of AMPK (Perfetti and Merkel, 2000; Gomez et al., 2002; Hayes et al., 2011b). However, a limitation of previous studies examining CNS GLP-1R signaling from hindbrain tissue is that one cannot distinguish between CNS cell types with results from tissue lysates. In addition, GLP-1Rs are expressed widely throughout the CNS (Merchenthaler et al., 1999) and in recent years GLP-1R signaling in several nuclei has been shown to regulate energy balance control (Alhadeff et al., 2012; Mietlicki-Baase et al., 2013a; Alhadeff et al., 2014; Alhadeff and Grill, 2014; Mietlicki-Baase et al., 2014; Secher et al., 2014; Hsu et al., 2015; Alhadeff et al., 2016). Collectively, these data highlight the importance of further examination of the specific cell types that bind GLP-1 and long acting GLP-1R agonists, the intracellular signaling cascades engaged within these cells, and the specific brain regions involved in GLP-1R-mediated control of energy balance.

Though PPG neurons in the NTS have been shown to project to mesolimbic and hypothalamic nuclei and exert control over energy balance (Larsen et al., 1997; Dossat et al., 2011; Alhadeff et al., 2012; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014; Hsu et al., 2015; Kanoski et al., 2016; Schmidt et al., 2016; Terrill et al., 2016), other nuclei throughout the CNS also express GLP-1R (Merchenthaler et al., 1999) but are under-investigated for their role in energy balance control. One of these nuclei is the lateral dorsal tegmental nucleus (LDTg), which integrates information from nuclei throughout the neuraxis to modulate motivated behavior (Cornwall et al., 1990; Schmidt et al., 2009; Shabani et al., 2010; Lammel et al., 2012; Shinohara et al., 2014; Steidl et al., 2015; Steidl et al., 2017). The LDTg is also critically positioned to integrate energy balance information throughout the brain (Cornwall et al., 1990) and thus may potentially mediate energy balance.

The lateral dorsal tegmental nucleus: an understudied hub potentially mediating motivated behavior and energy balance

Given that the control of energy balance is distributed throughout the CNS (Grill and Kaplan, 2002; Grill and Hayes, 2012), a deeper understanding of the relevant CNS circuitry and how it responds to and integrates energy balance signals to control food intake and body weight is needed. The LDTg of the mesopontine tegmentum represents an understudied hub that may mediate motivated behavior and energy balance. Historically, lesion studies established a role for the LDTg in motivated behavior (Blaha et al., 1996; Laviolette et al., 2000; Forster et al., 2002b; Alderson et al., 2005; Dobbs and Cunningham, 2014; Steidl et al., 2015) and recently more specific techniques have clarified these roles of the LDTg in motivated behavior and energy balance regulation (Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Schmidt et al., 2009; Dickson

et al., 2010; Shabani et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012; Lammel et al., 2012; Shinohara et al., 2014; Steidl et al., 2015; Steidl et al., 2017). The LDTg also expresses receptors for a variety of energy balance-relevant hormones, including GLP-1, amylin, ghrelin, oxytocin, orexin and PYY (Sexton et al., 1994; Merchenthaler et al., 1999; Parker and Herzog, 1999; Greco and Shiromani, 2001; Gould and Zingg, 2003; Dickson et al., 2011; Cabral et al., 2013). Indeed, the neuroanatomical connectivity of the LDTg with a variety of key feeding-relevant CNS nuclei and the expression of receptors for various feeding-related signals underscore the potential relevance of the LDTg as a hub for energy balance control.

Neuroanatomy of the LDTg: projection pathways and cellular phenotypes

Inputs to the LDTg include nuclei relevant for motivated behavior and energy balance such as the prefrontal cortex, lateral hypothalamus, lateral habenula, interpeduncular nucleus, VTA, substantia nigra pars compacta, parabrachial nucleus, and NTS (Satoh and Fibiger, 1986; Cornwall et al., 1990). The majority of these projections are from the ipsilateral region, but retrograde labeling from the lateral habenula, interpenducular nucleus, and NTS was found to be bilateral (Cornwall et al., 1990). Importantly, the NTS neurons that project to the LDTg are found at the level of the obex (Cornwall et al., 1990), which is the same rostral-caudal location of PPG neurons (Han et al., 1986; Larsen et al., 1997), suggesting that NTS PPG neurons may project to the LDTg. The specific neurochemical and neuropeptide phenotypes of most of these inputs require further investigation.

Efferent projection targets from the LDTg include the prefrontal cortex, hippocampus, lateral septum, thalamic nuclei, lateral habenula, lateral hypothalamus,

substantia nigra pars compacta, VTA, interpeduncular nucleus, dorsal and medial raphe, pedunculopontine tegmental nucleus, parabrachial nucleus, and the NTS (Satoh and Fibiger, 1986; Cornwall et al., 1990). Again, the majority of these projections are ipsilateral, but anterograde labeling in the prefrontal cortex, lateral septum, thalamic nuclei, lateral habenula, and VTA is bilateral (Cornwall et al., 1990). Among these outputs, the projection pathway from the LDTg to VTA has been the focus of a majority of scientific investigation on the LDTg (detailed below).

Three distinct subpopulations of LDTg neurons have been identified based on their neurochemical phenotype. Around 22% of LDTg neurons express choline acetyltransferase (ChAT) and are cholinergic, while around 38% of LDTg neurons express the glutamatergic marker vesicular glutamate transporter (vGlut2) and 40% of LDTg neurons express the gamma-aminobutyric acid (GABA)-ergic marker glutamic acid decarboxylase (GAD) (Wang and Morales, 2009). Importantly, only a small percentage of cholinergic neurons express vGlut2 or GAD, suggesting that these neuronal subtypes are predominately distinct populations of cells that are distributed throughout the LDTg (Wang and Morales, 2009). Though increasing evidence suggests a role for LDTg modulation of VTA dopaminergic transmission (detailed below), LDTg efferents to other nuclei that may play roles in motivated behavior and energy balance require further investigation.

The LDTg: discoveries with lesion studies

Specific lesions to the LDTg result in decreased spontaneous locomotor activity, blunted locomotor activity in response to amphetamine, methamphetamine, scopolamine, nicotine, and morphine, and increased latency to self-administer cocaine

(Blaha et al., 1996; Laviolette et al., 2000; Forster et al., 2002b; Alderson et al., 2005; Dobbs and Cunningham, 2014; Steidl et al., 2015). LDTg lesions also attenuate dopamine efflux in the NAc following VTA acetylcholinesterase inhibition (Blaha et al., 1996). Electrical stimulation of the LDTg activates VTA acetylcholine and glutamate receptors, leading to an increase in dopamine release in the NAc (Forster and Blaha, 2000; Forster et al., 2002a; Lester et al., 2010). Collectively, these previous studies established a role for the LDTg in modulating the mesolimbic dopamine system and have led the way for more targeted techniques that examine the specific mechanisms underlying how the LDTg modulates the dopamine system in motivated behaviors.

The LDTg: modulation of VTA dopaminergic system

The LDTg is required for burst firing of VTA dopamine neurons (Lodge and Grace, 2006). Neuroanatomical techniques show that the LDTg neurons synapse on NAc-projecting and prefrontal cortex-projecting VTA dopamine neurons as well as VTA GABA neurons, suggesting that the LDTg may have bi-directional control over dopamine release in the NAc and prefrontal cortex (Omelchenko and Sesack, 2005). However, the precise neurochemical phenotype of these projections needs to be further investigated, in terms of the percentage of LDTg cholinergic, glutamatergic, and GABAergic neurons that synapse onto each VTA neuronal subtype.

Behavioral techniques show that the LDTg is important for motivated behaviors, including drug-related behaviors. Hyperpolarizing LDTg neurons with carbachol prevents CPP for cocaine while pharmacological blockade of LDTg AMPA and N-methyl-D-aspartate (NMDA) receptors attenuates CPP for cocaine (Shinohara et al., 2014). In addition, rats receiving a microinjection of a muscarinic acetylcholine 2 receptor

antagonist in the LDTg show decreased responding for cocaine on progressive ratio schedules of reinforcement (Shabani et al., 2010). The LDTg also plays a role in cocaine seeking as AMPA receptor blockade in the LDTg attenuates cocaine priming-induced reinstatement (Schmidt et al., 2009). Current techniques employing optogenetics show that optogenetic excitation of the LDTg-to-VTA glutamatergic and cholinergic projection elicits a CPP (Lammel et al., 2012; Steidl et al., 2017). Furthermore, rats lever press for photostimulation of VTA-projecting LDTg axons (Steidl and Veverka, 2015). Collectively, research has established that the LDTg modulates the dopaminergic system, which has important implications in motivated behavior. These studies have focused on drug-related behaviors, but given the overlap in neural circuitry underlying drug abuse and feeding (Narayanan et al., 2010; DiLeone et al., 2012), this strongly suggests that the LDTg may be critical for the control of energy balance.

The LDTg: neuroendocrine signals

The LDTg expresses receptors for a variety of energy balance-relevant signals, including GLP-1, amylin, ghrelin, oxytocin, orexin and PYY (Sexton et al., 1994; Merchenthaler et al., 1999; Parker and Herzog, 1999; Greco and Shiromani, 2001; Gould and Zingg, 2003; Dickson et al., 2011; Cabral et al., 2013). Among these signals, ghrelin's action in the LDTg has been investigated to the greatest extent. Ghrelin administration into the LDTg increases acetylcholine release in the VTA, dopamine release in the nucleus accumbens, and ultimately food intake (Jerlhag et al., 2007; Dickson et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012). These data suggest that the LDTg can respond to ghrelin to control energy balance. However, whether other neuroendocrine signals act in the LDTg to regulate energy balance and how this might

impact downstream activity in the VTA or other LDTg projection targets requires further investigation. Given that the LDTg receives projections from the NTS at the level of the obex (the same rostral-caudal level of PPG neurons) and the LDTg expresses GLP-1R mRNA (Cornwall et al., 1990; Merchenthaler et al., 1999), I hypothesize that GLP-1R signaling in the LDTg modulates energy balance (see Figure 1.1).

It is crucial to examine CNS sites such as the LDTg that express a variety of neuroendocrine receptors and therefore potentially respond to and integrate a number of energy status signals. The LDTg binds amylin (Sexton et al., 1994), which is a pancreatic and centrally-derived anorectic hormone considered to be a promising target for obesity treatment (Aronne et al., 2007; Dunican et al., 2010; Singh-Franco et al., 2011; Hay et al., 2015; Mietlicki-Baase, 2016). Though amylin is traditionally thought to act in the area postrema (Lutz et al., 1998b; Lutz et al., 2001; Lutz, 2010b, a; Potes and Lutz, 2010; Potes et al., 2010; Potes et al., 2012; Roth et al., 2012), recent data has highlighted a broader role for amylin's action in the CNS to regulate energy balance with action in the VTA and NAc (Mietlicki-Baase et al., 2013b; Baisley and Baldo, 2014; Mietlicki-Baase et al., 2015b) and has shown that amylin can interact with other feeding related hormones (Lutz, 2013). Given that the LDTg binds amylin and projects to the VTA (Cornwall et al., 1990; Sexton et al., 1994), I hypothesize that amylin receptor signaling in the LDTg modulates energy balance and motivated behavior (see Figure 1.1).

Overview of dissertation

Given that energy balance control is distributed throughout the CNS and peripherally- or centrally-derived neuroendocrine signals can act at nuclei throughout the

brain [see (Grill and Kaplan, 2002; Grill and Hayes, 2012) for review], it is critical to examine the specific nuclei and cell types that are activated by these neuroendocrine signals and clinically relevant analogs. As GLP-1 and amylin regulate energy balance and are of clinical relevance, this thesis dissertation will investigate the cell specificity of GLP-1R signaling in the NTS for energy balance control and the LDTg as an understudied hub mediating energy balance via GLP-1 and amylin.

Recent attention has focused on the role of glutamatergic signaling in mediating the hypophagic effects of central GLP-1R activation (Hisadome et al., 2011; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014). Surprisingly, the literature has overlooked the potential contribution of astrocytes, a key cell population that regulates synaptic glutamate levels (Perego et al., 2000; Rimmele and Rosenberg, 2016). Data have shown that astrocytes within the CNS express GLP-1Rs, though this has not been examined in the context of energy balance (Chowen et al., 1999; Iwai et al., 2006; Kobayashi et al., 2013). In Chapter 2, I test the hypothesis that GLP-1Rs are expressed on astrocytes in the NTS and are relevant for energy balance control.

Projections from the NTS to the LDTg are understudied, particularly in the context of GLP-1 signaling. Given that the NTS produces GLP-1 and the LDTg expresses GLP-1R mRNA, there is strong rationale to evaluate the behavioral and physiological effects of GLP-1R activation in the LDTg (Cornwall et al., 1990; Merchenthaler et al., 1999). In Chapter 3, I test the hypothesis that GLP-1R signaling within the LDTg contributes to energy balance control by examining the pharmacological and physiological relevance of LDTg GLP-1Rs (see Figure 1.1).

The LDTg also expresses receptors for other neuroendocrine signals, including the core component of the amylin receptor, the calcitonin receptor (Sexton et al., 1994). In Chapter 4, I therefore test the hypothesis that amylin receptor signaling the LDTg contributes to energy balance control by examining the pharmacological and physiological relevance of LDTg amylin receptors (see Figure 1.1).

The data presented in this dissertation provide evidence that NTS astrocytes express GLP-1Rs, are relevant for energy balance control, and that GLP-1 and amylin each act in the LDTg to modulate energy balance. Collectively, these data broaden our understanding of the CNS action of neuroendocrine signals for energy balance control, in terms of both cell- and nuclei-specificity, providing greater insight into possible targets for the developing improved pharmacotherapies to treat obesity.

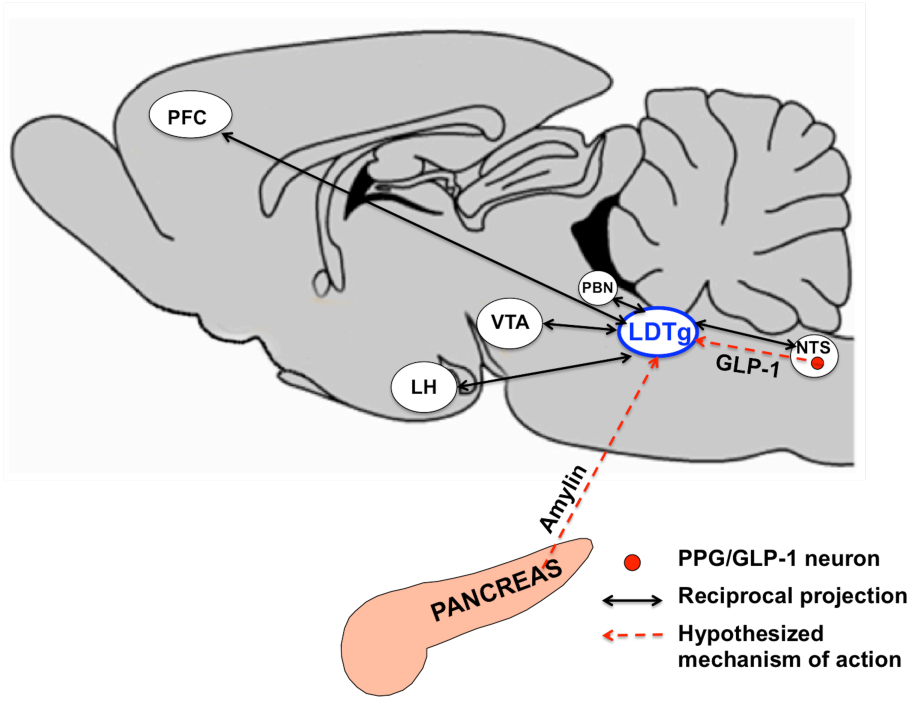

Figure 1.1. The LDTg represents a potential hub mediating energy balance. The LDTg sends reciprocal projections to multiple nuclei of relevance to energy balance control, including but not limited to the lateral hypothalamus (LH), nucleus tractus soliatrius (NTS), parabrachial nucleus (PBN), prefrontal cortex (PFC), and ventral tegmental area (VTA) (Cornwall et al., 1990). The LDTg expresses the GLP-1R (Merchenthaler et al., 1999) and the core component of the amylin receptor (Sexton et al., 1994). We hypothesize that NTS PPG/GLP-1 neurons project directly to the LDTg to control feeding and that the pancreatic-derived hormone amylin controls energy balance, in part, by direct action in the LDTg.

CHAPTER 2: Astrocytes Regulate Glucagon-Like Peptide-1 Receptor-Mediated Effects On Energy Balance

David J. Reiner[1]*, Elizabeth G. Mietlicki-Baase[1]*, Lauren E. McGrath[1], Derek J. Zimmer[1,2], Kendra K. Bence[2], Gregory L. Sousa[1,2], Vaibhav R. Konanur[3], Joanna Krawczyk[1], David H. Burk[4], Scott E. Kanoski[3], Gerlinda E. Hermann[4], Richard C. Rogers[4], and Matthew R. Hayes[1]

[1]Translational Neuroscience Program, Department of Psychiatry, Perelman School of Medicine

[2]Department of Animal Biology, School of Veterinary Medicine at the University of Pennsylvania, Philadelphia, PA, 19104, USA

[3]Department of Biological Sciences, Human and Evolutionary Biology Section, University of Southern California, Los Angeles, CA, 90089, USA

[4]Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, LA, 70808, USA

**These authors contributed equally to this work.*

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Abstract

Astrocytes are well-established modulators of extracellular glutamate, but their direct influence on energy balance-relevant behaviors is largely understudied. As the anorectic effects of glucagon-like peptide-1 receptor (GLP-1R) agonists are partly mediated by central modulation of glutamatergic signaling, we tested the hypothesis that astrocytic GLP-1R signaling regulates energy balance in rats. Peripheral administration of a fluorophore-labeled GLP-1R agonist, exendin-4, localizes within astrocytes and neurons in the nucleus tractus solitarius (NTS), a hindbrain nucleus critical for energy balance control. This effect is GLP-1R-mediated, as uptake of systemically administered fluorophore-tagged exendin-4 was blocked by central pretreatment with the competitive GLP-1R antagonist exendin-(9-39). *Ex vivo* analyses show prolonged exendin-4-induced activation (live cell calcium signaling) of NTS astrocytes and neurons; these effects are also attenuated by exendin-(9-39), indicating mediation by the GLP-1R. *In vitro* analyses show that application of GLP-1R agonists increases cAMP in astrocytes. Immunohistochemical analyses reveal that endogenous GLP-1 axons form close synaptic apposition with NTS astrocytes. Finally, pharmacological inhibition of NTS astrocytes attenuates the anorectic and body weight-suppressive effects of intra-NTS GLP-1R activation. Collectively, data demonstrate a role for NTS astrocytic GLP-1R signaling in energy balance control.

Introduction

The nucleus tractus solitarius (NTS) of the caudal brainstem is a critical nucleus in the regulation of energy balance. The NTS acts as a sensor for circulating neuroendocrine factors important for the control of food intake and is the first nucleus within the brain to receive and process vagally-mediated satiation signals arising from the gastrointestinal (GI) tract (Grill and Hayes, 2012). The NTS is therefore well-positioned as a key hub to combine a variety of feeding-related signals, and to transmit this integrated information to downstream nuclei to influence overall energy balance control (Grill and Hayes, 2009). Within the NTS, the incretin hormone glucagon-like peptide-1 (GLP-1) acts to control food intake and body weight (Grill and Hayes, 2012; Hayes et al., 2014a). Produced peripherally by intestinal L cells and centrally by preproglucagon (PPG) neurons within the caudal NTS, GLP-1 and GLP-1 receptor (GLP-1R) agonists suppress food intake and body weight through direct GLP-1R signaling in the NTS, as well as through action in other distributed nuclei in the brain (McMahon and Wellman, 1998; Grill and Hayes, 2009; Dossat et al., 2011; Alhadeff et al., 2012; Mietlicki-Baase et al., 2013a; Rupprecht et al., 2013; Alhadeff et al., 2014; Mietlicki-Baase et al., 2014; Secher et al., 2014; Hsu et al., 2015). However, the cellular and molecular mechanisms mediating the energy balance effects of GLP-1R activation remain largely under-investigated. GLP-1R agonists such as exendin-4 (Ex-4) and liraglutide are widely used in the treatment of type 2 diabetes mellitus (T2DM) (Hayes et al., 2014a), and liraglutide was recently approved by the FDA for the treatment of obesity (Tella and Rendell, 2015). These important medical applications of GLP-1R agonists highlight the urgent need to understand more fully the mechanisms by which GLP-1R

activation impacts physiology and behavior, including the control of feeding and body weight.

Vagal afferents synapsing in the NTS, as well as NTS PPG neurons themselves, are glutamatergic (Ritter, 2004; Grill and Hayes, 2012; Zheng et al., 2014). Thus, recent attention has focused on the role of glutamatergic signaling in mediating the anorexigenic effects of central GLP-1R activation. In particular, several studies demonstrate a role for the ionotropic AMPA/kainate glutamate receptors (Hisadome et al., 2011; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014) as downstream mediators of the food intake- and body weight-suppressive effects of GLP-1R signaling within mid- and forebrain nuclei. Importantly, these reports also indicate that the relevant GLP-1Rs are located presynaptically to AMPA/kainate receptors and can influence presynaptic glutamate release (Acuna-Goycolea and van den Pol, 2004; Amato et al., 2010; Hisadome et al., 2011; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014). However, to date, the ability of GLP-1R activation to affect energy balance via non-neuronal modulation of synaptic glutamatergic signaling has not been systematically evaluated.

Most studies of the central control of energy balance have focused on the actions of neurons, but astrocytes represent another key central nervous system (CNS) cell population that modulates glutamatergic neurotransmission, thereby potentially influencing behavior (Scofield and Kalivas, 2014; Yang et al., 2015). Indeed, glutamate is predominately cleared from the synapse by two subtypes of astrocytic glutamate reuptake transporters: glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST) (Perego et al., 2000; Danbolt, 2001). Intriguingly, a small body of

literature suggests that astrocytes within the CNS express the GLP-1R (Chowen et al., 1999; Iwai et al., 2006; Kobayashi et al., 2013), yet no studies have systemically examined its expression or behavioral relevance for energy balance control. As the NTS expresses GLP-1R (Hayes et al., 2010; Hayes, 2012) and is the first central site to receive and process within-meal vagally-mediated glutamatergic signals arising from the GI tract (Moran, 2006; Grill and Hayes, 2009), a combination of *in vitro*, *ex vivo*, and *in vivo* techniques was utilized to test the hypothesis that GLP-1R signaling in NTS astrocytes is functionally relevant for energy balance control.

Materials and Methods

Animals

Male Sprague-Dawley rats (320-420 g, Charles River) individually housed in hanging wire cages maintained at 23°C with a 12h light/12h dark cycle were used for the majority of these studies. For the live cell calcium imaging studies, male Long-Evans rats (250-300 g, breeding colony at Pennington Biomedical Research Center) were used. All procedures were approved by Institutional Animal Care and Use Committees (University of Pennsylvania, University of Southern California, Pennington Biomedical Research Center) and were performed according to the guidelines determined by the National Institutes of Health. Rats had *ad libitum* access to rodent chow and water unless otherwise noted.

Stereotaxic surgery

For behavioral experiments, rats were surgically implanted with cannula targeting either the medial NTS (mNTS) or 4th cerebroventricle (4th i.c.v.). Rats were anesthetized with an intramuscular injection of ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) and placed in a stereotaxic apparatus. Guide cannulas (Plastics One; 26-gauge) were implanted using the following coordinates: 4th i.c.v. (midline, 2.5 mm anterior to the occipital suture, internal cannula aimed 7.2 mm ventral to skull); mNTS (bilateral cannula; 1.0 mm posterior to occipital crest, ±0.5 mm lateral to midline, internal cannula aimed 8.7 mm ventral to skull). Bone screws and dental cement adhered the cannula to the skull surface. Animals were treated with analgesia (2 mg/kg meloxicam, s.c.) and allowed one week to recover. Behavioral testing began ~14 days after surgery.

Fourth i.c.v. cannula placements were verified prior to behavioral testing by assessing the hyperglycemic response to 5-thio-D-glucose (210 µg) as described (Hayes et al., 2009). mNTS injection sites were confirmed anatomically following a postmortem injection of 100 nl pontamine sky blue. Only animals passing functional or histological verifications were included in the final statistical analysis.

Immunohistochemical analyses

Detection and cellular localization of fluorescently-labeled Ex-4.

Rats were injected with fluorophore-labeled Ex-4 (fluoro-Ex-4; AnaSpec) intraperitoneally (10 µg/ml/kg; given ad libitum access to chow, vegetable shortening, and 30% sucrose). This ligand has been shown to bind to GLP-1R in cultured GLP-1R-expressing pancreatic beta cells (Rajan et al., 2015). Three hours after injection, rats were transcardially perfused with 4% paraformaldehyde in PBS followed by cryoprotection in 20% sucrose. This time point was selected based on previous research showing that the anorectic effects of central Ex-4 do not develop until 3 h post-administration (Hayes et al., 2011b; Kanoski et al., 2011; Kanoski et al., 2012a). Brains were sectioned on a cryostat in the coronal plane at 30 µm. Hindbrain sections were collected at the level of the area postrema (from bregma, AP -13.6 mm to -14.2 mm). Briefly, sections were blocked in 0.1 M PBS containing 5% normal donkey serum and 0.2% Triton-X at room temperature. Sections were incubated in primary antibodies overnight, and then following a PBS rinse, they were incubated in secondary antibodies for 2 h. Primary antibodies used were rabbit anti-NeuN (ab177487, 1:1000, Abcam) and goat anti-GFAP (ab53554, 1:1000, Abcam). Secondary antibodies (Jackson Immunoresearch) were donkey anti-goat Alexa Fluor 594 (1:500) and donkey anti-rabbit AMCA (1:200; Figure 2) or donkey anti-rabbit Alexa Fluor 647 (1:500; Figure 3).

Sections were visualized with a Leica SP5 X confocal microscope using the 20x and 63x oil immersion objectives and the 405, 488, and 594 laser lines. Image z-stacks with the 63x oil immersion were collected with a step size of 1 µm, while 2-3x optical zoom z-stack images using the same objective were collected with a step size of 0.5 µm. All images were collected sequentially to avoid contamination of signals from other fluorophores. Three-dimensional rotational animations were rendered from the collected z-stack images using Imaris 8.1.2 (Bitplane). To generate these animations, a Gaussian blur was first applied to the green channel (fluoro-Ex-4) to smooth the background using default settings (filter size 0.174 µm). For the blue channel (NeuN), a median filter (3 x 3 x 1 pixels) was run. Finally, the thresholds and gamma settings were adjusted [Green: thresholds = 4-40, gamma 2.5; Blue: thresholds = 2-90, gamma 3; Red (GFAP): thresholds = 2-200, gamma 1.2].

To evaluate whether the uptake of fluoro-Ex-4 into cells is GLP-1R-mediated, separate rats were given a 4th i.c.v. pretreatment with either the competitive GLP-1R antagonist exendin-(9-39) (Ex-9, 100 µg, American Peptide) or vehicle [2 µl artificial cerebrospinal fluid (aCSF; Harvard Apparatus)] 20 min before ip injection of fluoro-Ex-4 (10 µg/ml/kg). Rats were transcardially perfused 3 h later (4% paraformaldehyde in PBS followed by cryoprotection in 20% sucrose), and brains were immunohistochemically processed for NeuN, GFAP, and the fluorescently-tagged Ex-4 as described above. Three-dimensional rotational animations were generated for Ex-9-treated sections as described above. To evaluate colocalization of fluoro-Ex-4 in neurons and astrocytes from animals in each treatment group, z-stacks were obtained from 63x with a 2-3x optical zoom (vehicle: 5 z-stacks from 3 animals; Ex-9: 10 z-stacks from 3 animals) and colocalization analysis was performed in Imaris (version 7.6.5). The Gaussian filter was

applied to the green (Fluoro-Ex-4) channel using default settings (0.175 µm), the median filter was applied to the red (GFAP) and blue (NeuN) channels (3 x 3 x 1 px), and an additional Gaussian filter was applied to the blue (NeuN) channel (0.175 µm). The Imaris Colocalization module was then run using manual threshold settings (Green 4, Red 5, Blue 2) to generate a colocalization channel and statistics were exported. For each treatment (vehicle or Ex-9), separate Pearson's correlation coefficients (PCCs) were generated for colocalization of green and red (Fluoro-Ex-4 in astrocytes) and green and blue (Fluoro-Ex-4 in neurons).

Triple-labeling of GLP-1/GFAP/synaptophysin. Rats were perfused transcardially with ice-cooled 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borate buffer of pH 9.5. The brains were removed and immersed in fresh fixative containing 12% sucrose for 20–24 h at 4ºC. The brains were then blocked transversely at the level of the caudal midbrain, and each block was flash-frozen in dry-ice cooled hexane before being sectioned frozen on a sliding microtome (transverse plane, 30 µm thickness, 5 series). Hindbrain sections were collected at the level of the area postrema (from bregma, AP -13.6 mm to -14.2 mm). Sections were stored in antifreeze solution at -20°C until further processing.

For simultaneous immunohistochemical detection of GLP-1, GFAP, and synaptophysin, all washes and incubations were completed at room temperature unless otherwise noted. All primary and secondary antibodies were diluted in a solution of KPBS containing 2% donkey serum and 0.02% sodium azide. Three primary antibodies (rabbit anti-GLP-1, goat anti-GFAP, and mouse anti-synaptophysin) were used for this study. For each primary antibody used, a standard control was performed in which the

primary antibody was omitted from the protocol (to control for non-specific signal resulting from subsequent antibody and signal-detection reagents).

Tissue was washed with 0.02 M potassium phosphate-buffered saline (KPBS) to remove antifreeze, and then sections were permeabilized with 0.3% Triton X-100 in KPBS for 30 min. After KPBS washes, the tissue went through a blocking step of 30 min in 2% normal donkey serum at room temperature. Tissue was incubated overnight (~18 h) at 2ºC in rabbit anti-GLP-1 antibody (T-4363, 1:5000, Peninsula Labs). After KPBS washes, tissue was incubated for 48 h with goat anti-GFAP (ab53554, 1:500, Abcam) and mouse anti-synaptophysin (61012, 1:1000, PROGEN Biotechnik). Next, sections were washed and then incubated overnight at 2ºC in donkey anti-rabbit secondary-Cy3 conjugate (1:500, Jackson Immunoresearch), donkey anti-goat secondary-AMCA conjugate (1:500, Jackson Immunoresearch), and donkey anti-mouse secondary-AF488 conjugate (1:500, Jackson Immunoresearch). Sections were mounted onto glass slides and coverslipped using 50% glycerol in KPBS mountant. Photomicrographs were acquired as optical slices using a Zeiss LSM 700 UGRB confocal system controlled by Zeiss Zen software.

Live cell calcium imaging

To prepare brainstem sections for live cell calcium imaging experiments, the dyes Calcium Green-1 AM (CG; Life Technologies) and sulforhodamine 101 (SR101; Molecular Probes) were injected into the NTS of rats (n=6) as previously described (Hermann et al., 2009; Vance et al., 2015). CAG is a calcium reporter dye that labels both neurons and astrocytes, while SR101 labels only astrocytes (McDougal et al., 2011), allowing us to discriminate between astrocytes and neurons in our imaging recordings. Cellular uptake of these dyes occurred within 30-45 min. The anesthetized

rats were decapitated, and the brainstem was quickly harvested. Pre-labeled brainstem was cut into coronal sections (300 μm) on a vibrating microtome (Leica VT1200) and submerged in a cold (4^{o}C) carbogenated (95% O_2 / 5% CO_2) cutting solution [detailed descriptions in (Vance et al., 2015)].

Live cell calcium imaging of prelabeled astrocytes and neurons was performed as previously described (Hermann et al., 2009; Vance et al., 2015). Hindbrain slices were placed in and acclimated to the recording chamber of a Nikon F1 fixed stage upright microscope with constant exposure to Krebs' recording solution. Each slice was exposed to only one of two experimental designs. In one experiment, the slice was exposed to 100 nM Ex-4 in the perfusion media for 80 s and responses were monitored for a minimum of 3 min. In the other experiment, the slice was pre-treated with the competitive GLP-1R antagonist Ex-9 [200 nM; (Schepp et al., 1994)] for 5 min and then exposed to Ex-4 for 3 min. For both designs, slices were then washed out with normal Krebs' for 10 min and next were stimulated by exposure to Krebs' recording solution containing 100 μM ATP and 500 μM L-glutamate for 80 s. This ATP/glutamate challenge was used as a control to confirm that the neurons and astrocytes were viable and capable of producing calcium signals in response to experimental agonists. Only those cells (astrocytes and neurons) that responded to the ATP/glutamate challenge were included in the response analysis. Our previous studies (Vance et al., 2015) have shown that response magnitudes of neurons or astrocytes to ATP/glutamate do not decline even after multiple exposures.

A Prairie Technologies (Bruker Corporation) line-scanning laser confocal head equipped with a Photometrics CoolSNAP HQ camera performed time-lapse laser

confocal calcium imaging. Nikon Elements AR software was used to analyze the confocal live cell fluorescent signals in the astrocytes and neurons as previously described (Hermann et al., 2009; Vance et al., 2015). Individual astrocytes and neurons were designated as regions of interest (ROI), and their fluorescence signal over time was captured. Background fluorescence was subtracted from the fluorescence signal. The relative changes in cytoplasmic calcium in the cells were expressed as changes in fluorescence [($\Delta F/F$)%], where F is the intensity of the baseline fluorescence signal before stimulation, and ΔF is the difference between the peak fluorescence intensity and the baseline signal. Minimum of 5% change in fluorescence per cell was required to be included in analysis (McDougal et al., 2011).

Intracellular cAMP signaling response of immortalized medullary rat astrocytes

Immortalized rat type-1 astrocyte DI-TNC1 cells (ATCC) were maintained at 37°C in 5% CO_2 and were cultured in 1x DMEM with with 4.5 mg/ml glucose (GIBCO/Invitrogen), 10% (v/v) fetal bovine serum (Thermo Fisher Scientific Inc.), and 2% penicillin (10,000 I.U./ml)-streptomycin (10,000 ug/ml) solution (Mediatech, Inc.). Separate plates of DI-TNC1 cells were treated with one of three GLP-1R agonists [GLP-1 (American Peptide), Ex-4 (American Peptide), or liraglutide (Bachem)]. Three hour serum-starved DI-TNC1 astrocytes, cultured in a 96-well culture plate (6 × 104 cells/well), were treated with three doses of each agonist (0.1, 0.5, or 1.0 nmol; 3 plates per agonist/dose combination) for 15 min. cAMP levels were calculated using a standard curve of fmol of cAMP/10^4 DI-TNC1 cells. The same vehicle treatment (n=9 plates) was used for comparison to each GLP-1R agonist.

Feeding behavior

Comparison of 4th i.c.v. Ex-4 versus fluorophore-labeled Ex-4. Approximately 15 min before dark cycle onset, *ad libitum*-fed rats (n=4) received an i.c.v. injection of Ex-4 (0.3 µg), fluorophore-labeled Ex-4 (0.3 µg), or vehicle (1 µl aCSF). Doses of Ex-4 were chosen based on the literature (Hayes et al., 2011b), and each rat received all treatments in a counterbalanced within-subjects design. At dark onset, rats were presented with pre-weighed chow and intakes were recorded to the nearest 0.1 g at 1, 3, 6, and 24 h post food presentation. Body weights were recorded at 0 and 24 h after injection. Injections were separated by a minimum of 72 h.

mNTS inhibition of astrocyte activity. Approximately 30 min before dark cycle onset, *ad libitum*-fed rats (n=5) received a unilateral intra-mNTS pretreatment of vehicle (100 nl 0.1 M PBS) or the astrocyte Krebs cycle inhibitor fluorocitrate (DL-fluorocitric acid barium salt, Sigma-Aldrich; 413 ng) followed by a second unilateral mNTS injection of aCSF (100 nl) or Ex-4 (0.05 µg) in a within-subject counterbalanced design. At dark onset, rats were presented with pre-weighed chow and intakes were recorded to the nearest 0.1 g at 1, 3, 6, and 24 h post food presentation. Body weights were recorded at 0 and 24 h after injection and injections were separated by a minimum of 72 h.

Statistical analyses

Data for all experiments are expressed as mean ± SEM, with significance set at $p<0.05$. To assess colocalization of fluoro-Ex-4 in NTS cells, PCCs for the different 4th i.c.v. drug conditions (vehicle or Ex-9) were compared by separate two-tailed t-tests for each cell type (neurons or astrocytes). The PCC for astrocyte colocalization in one z-stack from an Ex-9-treated animal was a statistical outlier (greater than 2 standard deviations from the mean) and that z-stack was eliminated from all statistical analyses.

In vitro cAMP data were analyzed by separate one-way ANOVAs, followed by Dunnett's post hoc analysis when the overall ANOVA was significant. For calcium imaging studies, data were evaluated using the Mann-Whitney U-test. Results of the feeding studies were analyzed by one-way or two-way mixed design ANOVA, with drug condition as a within-subjects factor, and comparisons between treatment groups were made by Dunnett's (4^{th} i.c.v.) or Student Neuman-Keuls (mNTS) post hoc tests. Analyses were performed using Statistica 12.0 or 13.0 (StatSoft).

Results

Astrocytes and neurons in the NTS express GLP-1Rs and internalize GLP-1R ligands

To provide anatomical evidence that GLP-1Rs are expressed on astrocytes in the NTS, we tested the uptake of a fluorophore-labeled version of the GLP-1R agonist exendin-4 (fluoro-Ex-4) by neurons and astrocytes in the NTS, given the lack of a validated commercially-available GLP-1R-selective antibody. First, we verified that fluoro-Ex-4 has a comparable pharmacokinetic profile of effect on food intake and body weight as an equal dose of unlabeled Ex-4 when administered 4th i.c.v. in rats (Figure 1; feeding, $F_{2,6}$=6.92, p=0.03; body weight gain, $F_{2,6}$=6.36, p=0.03; for both feeding and body weight gain, vehicle versus Ex-4 or fluoro-Ex-4, p<0.05, Ex-4 versus fluoro-Ex-4, p>0.05). Fluoro-Ex-4 was then injected systemically (10 µg/kg, i.p.) and the animals were transcardially perfused 3 h later. The brains were immunohistochemically processed to label astrocytes and neurons, in conjunction with visualization of fluoro-Ex-4. Confocal microscopy revealed co-localization of fluoro-Ex-4 in both GFAP-positive astrocytes and NeuN-positive neurons in the NTS following systemic delivery (Figure 2). Further, a 3-dimensional rotation video of 63x z-stack overlays within the NTS revealed that systemically delivered fluoro-Ex-4 was internalized by both NTS astrocytes and neurons (See Figure 2C for still from video). This movie avoids the possibility of false overlaps that may exist in the 2-dimensional image (e.g., fluoro-Ex-4 directly in front of or behind the cell may appear to be co-localized in a 2-dimensional image, but not in a 3-dimensional rotation).

To confirm that the binding and uptake of fluoro-Ex-4 in NTS cells is GLP-1R-mediated, we tested whether internalization of peripherally-administered fluoro-Ex-4 would be attenuated by pharmacological blockade of hindbrain GLP-1Rs. As shown in

the Figure 3A and similar to our first study, fluoro-Ex-4 was co-localized in both astrocytes and neurons in the NTS after 4th i.c.v. pretreatment with vehicle (2 µl). However, hindbrain injection of the competitive GLP-1R antagonist exendin-(9-39) (Ex-9; 100 µg) blocked uptake of fluoro-Ex-4 in NTS cells (Figure 3B and C). Quantitative analyses suggest that Ex-9 pretreatment blocks colocalization of fluoro-Ex-4 in both astrocytes [vehicle: r = 0.20±0.12; Ex-9: r = -0.07±0.03; t(12)=2.75, p=0.02] and neurons [vehicle: r = 0.23±0.10; Ex-9: r = 0.03±0.04; t(12)=2.36, p=0.04]. Although this analysis does not account for the location of the fluoro-Ex-4 (e.g., on the cell surface versus internalized), as Ex-9 is a competitive GLP-1R antagonist, it would likely block both receptor binding and internalization of the agonist. Collectively, these data provide further evidence that uptake of the fluorescently-tagged Ex-4, like untagged Ex-4, is mediated by the GLP-1R. Again, inclusion of a 3D rotational video (see Figure 3C for still) avoids the potential for false overlaps in the 2-dimensional image in Figure 3B.

These data provide novel anatomical evidence suggesting that astrocytes bind and take up GLP-1R ligands; these results also show that a peripherally-injected GLP-1R agonist can penetrate the blood-brain barrier and gain access to the NTS. Furthermore, these data show that a portion of astrocytes and neurons do not take up the fluoro-Ex-4, suggesting that these cells do not express the GLP-1R, while other cells in adjacent proximity show clear uptake of the fluoro-Ex-4 and putatively express GLP-1Rs (shown explicitly in Figure 2).

NTS astrocytes and neurons show increased live cell calcium signaling following Ex-4-induced GLP-1R activation

To test the ability of astrocytes in the NTS to respond to GLP-1R activation, *ex*

vivo live cell calcium signaling in NTS-containing rat brainstem slices was evaluated following bath application of Ex-4 (100 nM). Astrocytes were pre-labeled with the astrocyte-specific dye SR101, and both astrocytes and neurons were pre-labeled with the calcium-sensitive dye CalciumGreen-1 AM (CG; Figure 4A-C). An increase in intracellular calcium corresponded to an increase in fluorescence and is indicative of cellular activation (Rogers et al., 2006). In viability experiments, ATP/glutamate stimulation activated 129 NTS astrocytes with a mean magnitude of increased fluorescence of 24.6 $\pm$ 1.2% (Figures 4D, F, G). Of the 129 NTS astrocytes responsive to the ATP/glutamate challenge, 40% were also directly responsive to Ex-4 stimulation (Figure 4E, F) with a 20.1 $\pm$ 4.2% mean magnitude of response (Figure 4G), similar in magnitude to the response to ATP/glutamate. A similar proportion of NTS neurons were responsive to Ex-4 stimulation (14 out of 38 ATP/glutamate-responsive neurons; Figure 4E, F). Those neurons that were responsive to both stimuli averaged lower magnitudes in their response to Ex-4 relative to their evoked response to ATP/glutamate (Figure 4H).

In separate hindbrain slices, pre-exposure to the competitive GLP-1R antagonist Ex-9 reduced the overall number of Ex-4-responsive astrocytes from 40% to approximately 20% (Figure 4F). NTS astrocytes that were still activated by Ex-4 following Ex-9 pre-exposure had a significantly diminished response to Ex-4 (Figure 4G; $U=37$, $p=0.0002$). In contrast, while Ex-9 suppressed the number of NTS neurons responsive to Ex-4 from ~37% to 13% (Figure 4F), the magnitude of their response to Ex-4 was not further inhibited by Ex-9 (Figure 4H; $U=20$, $p=0.53$).

GLP-1R activation increases cAMP in astrocytes in vitro

Downstream intracellular signaling events of GLP-1R activation in neurons include elevated levels of cAMP and PKA, which are required for the anorexigenic effects of GLP-1R activation (Hayes et al., 2011b). However, it is unknown whether this signaling pathway is also recruited by astrocytic GLP-1R activation. To determine whether GLP-1R activation increases cAMP levels specifically in astrocytes, we measured cAMP concentration in an immortalized rat type-1 astrocyte cell line (DI-TNC1) after exposure to one of three GLP-1R agonists [GLP-1(7-36), exendin-4 (Ex-4), or liraglutide; 0.1, 0.5, 1.0 nmol]. These *in vitro* analyses demonstrated that each of the three GLP-1R ligands caused a significant dose-dependent increase in cAMP concentrations in astrocytes (Figure 5; GLP-1: $F_{3,14}$=3.74, p=0.04; Ex-4: $F_{3,14}$=6.02, p=0.01; liraglutide: $F_{3,14}$=6.12, p=0.01). These data suggest that like the cAMP responses previously observed in neurons (Hayes et al., 2011b), cAMP signaling is engaged downstream of GLP-1R activation in astrocytes.

Endogenous GLP-1-immunopositive fibers form synaptic contact with NTS astrocytes

To provide evidence of a potential endogenous source of GLP-1 to NTS astrocytes, immunohistochemical triple-labeling was performed to identify GLP-1 axons, astrocytes (GFAP-immunopositive), and presynaptic terminals (synaptophysin-immunopositive) in the NTS of rats. As shown in Figure 6, GLP-1 and synaptophysin co-localized in NTS GFAP-positive astrocytes, indicating that GLP-1 axons terminate in close apposition (presumably synaptic contacts) to NTS GFAP-positive astrocytes. These anatomical data suggest that NTS PPG neurons are a putative endogenous source of GLP-1 to NTS astrocytes.

Astrocytes mediate the food intake- and body weight-suppressive effects of GLP-1R activation in the NTS

To test whether NTS astrocytes play a role in the energy balance effects of GLP-1R activation, food intake and body weight gain was measured in rats following NTS-targeted treatment with the astrocyte Krebs cycle inhibitor fluorocitrate (Lian and Stringer, 2004; Hermann et al., 2014) (413 ng) and Ex-4 (0.05 µg). As shown in Figure 7, NTS pre-treatment with fluorocitrate attenuated both the hypophagia and body weight reduction produced by direct NTS administration of Ex-4 [24 h food intake: interaction between fluorocitrate and Ex-4, $F_{1,4}$=10.08, p=0.03; body weight: $F_{1,4}$=33.87, p=0.004; for both 24 h food intake and body weight, vehicle/vehicle vs. vehicle/Ex-4, p<0.05; vehicle/Ex-4 vs. fluorocitrate/Ex-4, p<0.05; no statistically significant interaction between fluorocitrate and Ex-4 at 1, 3, or 6 h (all $F_{1,4}$≤1.04, all p≥0.37; data not shown)]. Importantly, behavioral data from animals (n=5) with missed mNTS cannula placements does not show hypophagia after GLP-1R activation or an effect of fluorocitrate (See Figure A1 in Appendix; ($F_{1,4}$<3.6, p>0.13). These findings suggest that inhibiting astrocyte activity attenuates the ability of NTS GLP-1R activation to promote negative energy balance.

Discussion

Astrocytes play a critical role in the regulation of glutamatergic neurotransmission, yet are surprisingly understudied as potential mediators of energy balance-relevant signals. Given that the food intake- and body weight-suppressive effects of central GLP-1R signaling are mediated in part by presynaptic modulation of glutamate signaling (Hisadome et al., 2011; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014), it is intriguing to consider the idea that GLP-1Rs expressed on astrocytes may influence glutamate signaling to affect energy balance. Therefore, we focused our attention on astrocytes in the NTS because of the critical role this nucleus serves in processing vagally-mediated glutamatergic satiation signals (Grill and Hayes, 2012), as well as the endogenous relevance of NTS GLP-1R signaling for the normal control of food intake (Hayes et al., 2009). Novel data presented here show that: [1] astrocytes are activated by GLP-1 and GLP-1R agonists, [2] the GLP-1R agonist Ex-4 is internalized by and produces robust calcium signaling in NTS neurons and astrocytes, [3] the endogenous central GLP-1 neurons form close synaptic appositions with NTS astrocytes, and [4] NTS astrocyte activity is required for the hypophagia and reduction in body weight produced by NTS GLP-1R activation.

Unlike the short half-life of endogenous peripheral GLP-1, systemically administered GLP-1R agonists Ex-4 and liraglutide are resistant to degradation by DPP-IV and thus remain in circulation for longer periods of time to impact energy balance (Kanoski et al., 2011; Secher et al., 2014; Sisley et al., 2014). These drugs are used clinically for the treatment of T2DM, and more recently, liraglutide also has been FDA-approved for use in the treatment of obesity (Tella and Rendell, 2015), highlighting the importance of fully understanding the mechanisms by which these GLP-1R agonists

exert their metabolic effects. To address the ability of GLP-1R agonists to activate NTS astrocytes, an important first step was to confirm GLP-1R expression on astrocytes. Given the lack of a validated commercially-available GLP-1R-selective antibody, we instead availed of a novel use of fluorophore-tagged Ex-4 and tested its ability to label GLP-1R expressing neurons and astrocytes in the NTS. Injection of fluoro-Ex-4 peripherally revealed that some, but not all NTS astrocytes and neurons were labeled with the fluorescent tag, indicating binding of the ligand and thus probable GLP-1R expression. The finding that uptake of peripherally-administered fluoro-Ex-4 was blocked by hindbrain pretreatment with Ex-9 provides further support for GLP-1R mediation of these effects. These results also provide visual evidence that this long-lasting GLP-1R agonist can cross the blood-brain barrier and bind to receptors within the CNS. Perhaps most intriguing is the discovery that fluoro-Ex-4 was internalized by not only neurons but also astrocytes.

A combination of *in vitro* and *ex vivo* approaches was used to demonstrate that astrocytes not only internalize a GLP-1R agonist, but also are responsive to GLP-1R activation. *Ex vivo* live cell calcium imaging data indicated that subpopulations of astrocytes as well as neurons within the NTS are responsive to GLP-1R activation. Approximately 40% of all viable NTS astrocytes and neurons also responded to Ex-4. However, pre-exposure of the slice to the competitive GLP-1R antagonist Ex-9 reduced the numbers of NTS astrocytes and neurons responding to Ex-4. Furthermore, the magnitude of the NTS astrocyte response to Ex-4 was also reduced by Ex-9; in contrast, Ex-9 pretreatment did not further blunt the magnitude of response to Ex-4 in neurons that remained responsive. This observation opens the possibility that NTS neuron

responses to Ex-4 may actually be activated, at least in part, by an indirect gliotransmission driven pathway following GLP-1R activation of astrocytes.

To directly examine whether GLP-1R-mediated calcium signaling engages cAMP as a second messenger in astrocytes, an immortalized rat type-1 astrocyte DI-TNC1 cell line was utilized. Similar to the response previously observed in neuronal cultures (Hayes et al., 2011b), cAMP levels increased dose-dependently in the rat astrocyte cell line following activation by any one of three GLP-1R agonists tested (GLP-1, Ex-4, or liraglutide). Together, these data suggest that GLP-1R activation and recruitment of the appropriate intracellular signaling cascades occur not only in neurons but also in astrocytes.

Previous research has shown that endogenous NTS GLP-1R signaling is physiologically relevant for food intake control, as blockade of endogenous NTS GLP-1R by hindbrain administration of the GLP-1R antagonist exendin-(9-39) leads to a significant increase in food intake (Hayes et al., 2009). However, it is unknown whether this effect is due to blockade of GLP-1R expressed on NTS neurons, astrocytes, and/or vagal afferent terminals. Given that PPG neurons within the NTS synthesize GLP-1, endogenous GLP-1 produced within the NTS could engage astrocytes for energy balance control. Indeed, the novel immunohistochemical analyses presented here provide anatomical evidence that endogenous GLP-1 axons make close appositions with NTS astrocytes. This finding does not establish whether these points of contact are classical “synapses”, and it remains unclear whether true synaptic communication occurs at these apposed membranes or whether the GLP-1 axons and NTS astrocytes interact in some other way. Nevertheless, these close appositions are suggestive of an

interaction between the GLP-1-producing neurons and astrocytes, and future studies are therefore warranted to examine the contribution of endogenous, astrocyte-specific GLP-1R signaling for the control of energy balance.

Behavioral food intake and body weight analyses demonstrated that inhibition of NTS astrocyte metabolic function with fluorocitrate attenuated the intake- and body weight suppression produced by NTS GLP-1R activation. This finding provides support for the hypothesis that NTS astrocytes not only bind, internalize, and display appropriate signaling responses to GLP-1R agonists, but also that NTS astrocytes mediate the energy balance effects of GLP-1R signaling. Fluorocitrate inhibits glial cells by blocking aconitase activity, thereby disrupting the tricarboxylic acid (TCA) cycle (Peters, 1957; Brand et al., 1973; Fonnum, 1997). Although multiple types of glia can be inhibited by fluorocitrate, it appears to have the most potent effects on astrocytes (Paulsen et al., 1987). Any potential influence of oligodendrocytes or microglia on the behavioral effects observed in this experiment would likely be minimal in comparison to the contributions of astrocytes, but this possibility cannot be ruled out. It should also be noted that at very high doses, fluorocitrate can also disrupt neuronal activity (Koenig, 1969), but the dose used in our study (413 ng, or 0.5 nmol) is far below subthreshold concentrations for neuronal effects (Paulsen et al., 1987; Hassel et al., 1992; Willoughby et al., 2003).

As fluorocitrate blocks all astrocyte activity, it is impossible to tease apart the exact role of astrocytes in mediating GLP-1R intake suppression by this pharmacological manipulation. For example, we cannot definitively exclude the possibility that astrocytes are indirectly involved with the behavioral effects of GLP-1R activation. That is, NTS injection of fluorocitrate will non-selectively inhibit all NTS astrocytes, not only GLP-1R-

expressing astrocytes, so one cannot rule out the alternative explanation that astrocytes are also engaged along with or even downstream of neuronal GLP-1R activation, or that non-GLP-1R-expressing astrocytes may play some indirect role in these anorexic effects. However, the results of the calcium imaging studies argue against these possibilities. The near-simultaneous activation of NTS astrocytes and neurons by Ex-4 in that experiment supports the hypothesis that Ex-4 acts directly on at least a subset of NTS astrocytes. Even if GLP-1R activation in these astrocytes accompanies neuronal GLP-1R activation, our behavioral data suggest that astrocytes are required for the control of energy balance by NTS GLP-1R activation.

An important empirical question arising from the current studies is the mechanism by which astrocytic GLP-1R activation might alter glutamatergic neurotransmission to subsequently affect energy balance. Increased cAMP levels have been linked to reduced expression of glutamate transporters (Lim et al., 2005). Our data demonstrating that astrocytic GLP-1R activation elevates cAMP hints at the intriguing possibility that astrocytic glutamate transporters (e.g. GLT-1, GLAST) may be downregulated as a result of GLP-1R activation, providing a potential mechanism by which GLP-1R signaling in astrocytes could increase glutamate in the NTS synapse and consequently reduce food intake (Ritter, 2004; Hisadome et al., 2011; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014).

Collectively, the current data show that NTS astrocytes are engaged by GLP-1R activation to control energy balance. Central activation of GLP-1R in a variety of nuclei has a broad range of behavioral and physiological effects. In addition to the NTS, several nuclei within the brain mediate the hypophagic effects of GLP-1 [see (Hayes et

al., 2014a) for review]. Additionally, CNS GLP-1R mediate diverse phenomena including glycemic control (Sandoval et al., 2008), motivated behavior (Harasta et al., 2015), and even presumably protection against neurodegeneration (Bao et al., 2015). The possibility that astrocytic GLP-1R activation contributes to these effects is just beginning to be considered. As FDA-approved drugs such as exendin-4 and liraglutide target the central GLP-1 system, the present findings may also be of clinical significance as the scientific community strives to comprehend more fully the mechanisms by which GLP-1R agonists exert their physiological and behavioral effects. In summary, the current findings not only demonstrate the importance of NTS astrocytic GLP-1R signaling for the control of food intake, but also represent the beginning of an enormous opportunity for deeper understanding of the mechanisms by which central GLP-1 signaling influences physiology and behavior.

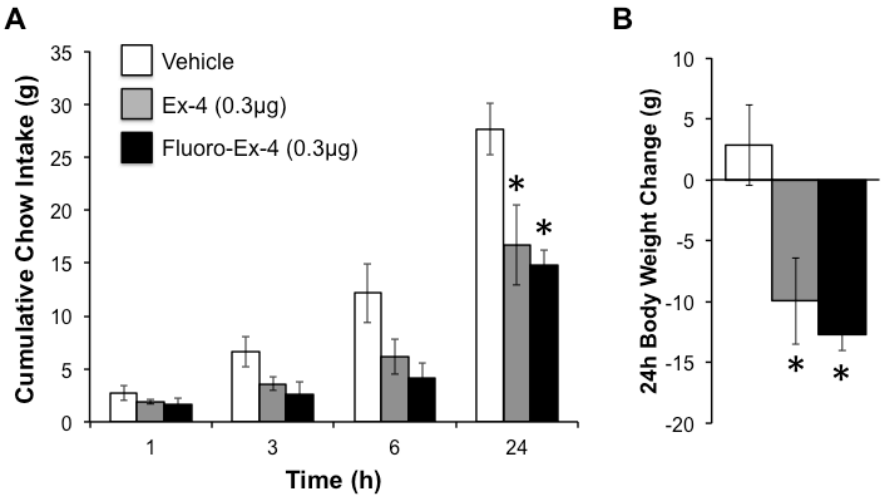

Figure 1. Hindbrain administration of fluorescently-labeled Ex-4 produces energy balance effects comparable to those of unlabeled Ex-4. Fourth i.c.v. administration of Ex-4 (0.3 µg) reduces chow intake (A) and body weight (BW) gain (B) in rats (n=4); an equal dose of fluorescently-labeled Ex-4 (Fluoro-Ex-4) given 4th i.c.v. causes similar suppression of feeding and body weight gain. The key for (a) applies to both panels. *, p<0.05 compared to vehicle. Data shown as mean ± SEM.

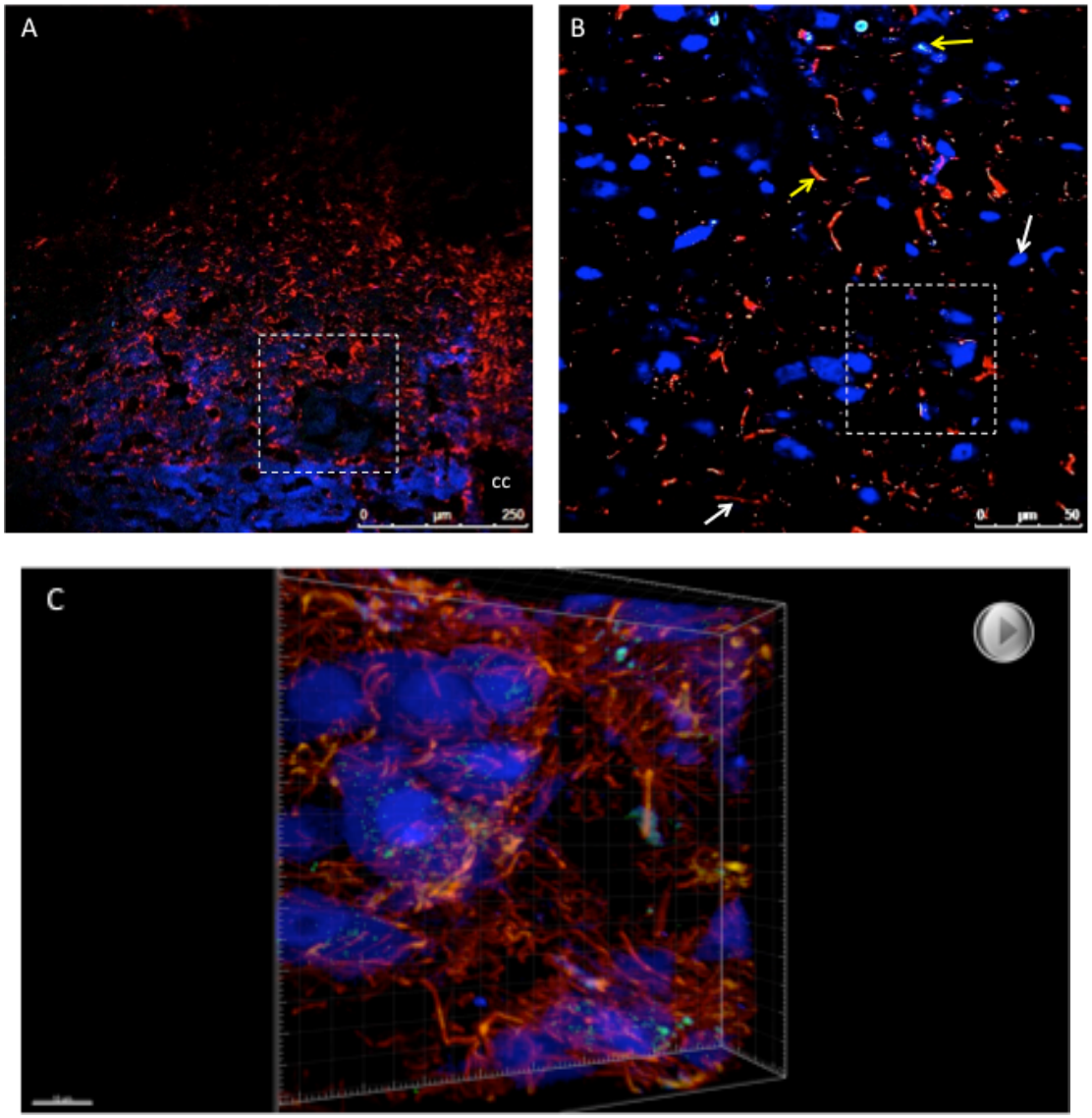

Figure 2. Fluorescently-labeled Ex-4 is taken up by neurons and astrocytes within the NTS following systemic administration. Rats (n=2) were injected with fluoro-Ex-4 peripherally (10 µg/kg, ip) and sacrificed 3 h later. A representative NTS-containing section is shown at 20x magnification in (a), with GFAP-positive cells shown in red and NeuN-positive cells in blue. The dotted box outlined in panel (A) is magnified in (B). This higher-magnification image in (B) depicts a single image within a z-stack (0.5 µm step size) taken with the 63x oil immersion objective shows fluoro-Ex-4 labeling in GFAP-positive cells (red) and NeuN-positive cells (blue). White arrows indicate cells lacking co-localization with fluoro-Ex-4, while yellow arrows indicate cells that take up fluoro-Ex-4. The dotted box in (B) indicates the field of view in (C), which shows a still from a representative three-dimensional rotational video of fluoro-Ex-4 co-localization in NTS astrocytes and neurons following systemic administration of fluoro-Ex-4. cc = central canal.

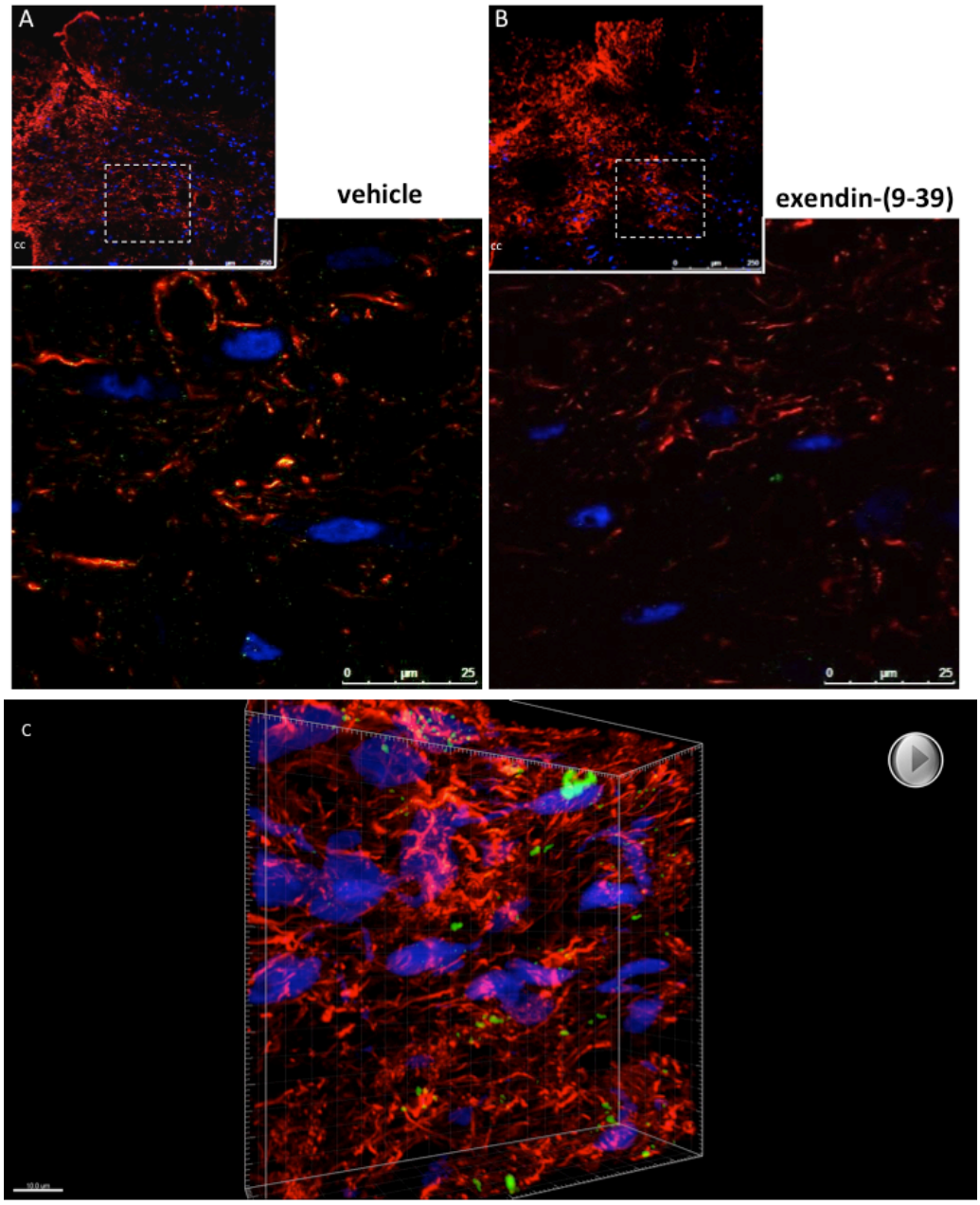

Figure 3. The internalization of peripherally administered fluoro-Ex-4 in NTS cells is blocked by 4th i.c.v. pretreatment with a GLP-1R antagonist. While uptake of fluoro-Ex-4 (10 μg/kg, ip) was observed in NTS astrocytes and neurons of rats given a 4th i.c.v. pretreatment with vehicle (a; n=3), central pretreatment with Ex-9 (100 μg) blocked this effect (b; n=4). Fluoro-Ex-4 is shown in green, GFAP-positive cells are shown in red, and NeuN-positive neurons are shown in blue. The smaller insets at the top of each panel depict representative NTS-containing coronal sections at 20x magnification. The boxed area of each inset was magnified to 63x and a further 2-3x optical zoom was applied to generate the large images. A still from the 3-dimensional rotational video from a subfield of B is shown in C. cc= central canal.

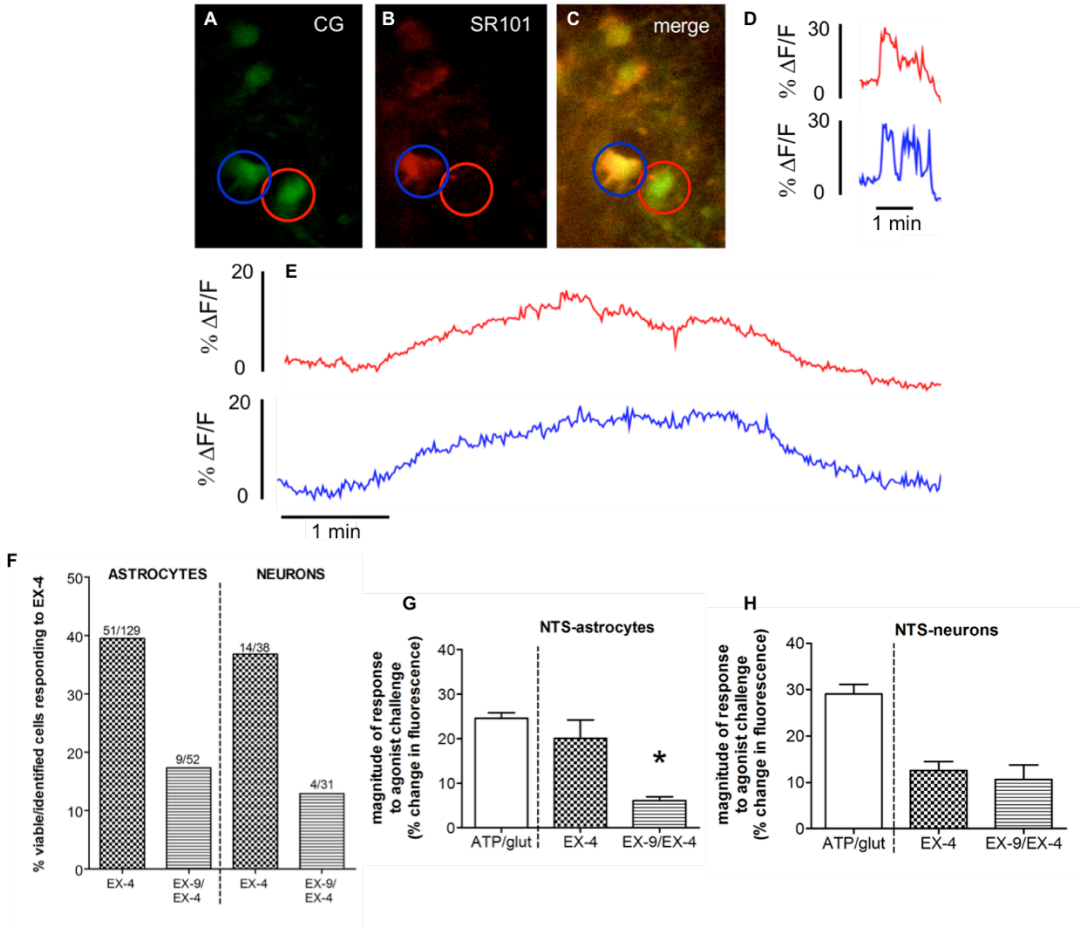

Figure 4. GLP-1R activation increases calcium signaling in subsets of both NTS astrocytes and neurons. NTS cells (n=6 rats) were preloaded with CalciumGreen-1 AM (calcium-sensitive dye; labels astrocytes and neurons) and SR101 (astrocyte-specific red dye); yellow colocalization confirms that the calcium-sensitive dye is localized in astrocytes (A-C). Astrocytes and neurons are designated as “regions of interest” (ROI); red = neuron, blue = astrocyte. Calcium-induced changes in fluorescence in response to perfusion parameters are quantitated over time. Percent change in fluorescence for each ROI over time is shown in corresponding line colors (neuron in red, astrocyte in blue). Control (viability) experiments were performed [representative traces in (D)], in which a cocktail of ATP/glutamate was applied for 80 sec to identify viable, responsive astrocytes and neurons (i.e., increased intracellular calcium as demonstrated by increased fluorescence). The increase in intracellular calcium is immediate due to the combined opening of ligand-gated cation channels and the intracellular release of stored calcium. Approximately 40% of NTS-astrocytes and neurons were also activated by GLP-1R agonist Ex-4 [representative traces in (E), quantification of cells in (f)]. Pre-exposure of the slice to the competitive GLP-1R antagonist Ex-9 reduces the number of Ex-4 responsive cells to approximately 20% (F). The subpopulation of NTS astrocytes activated by Ex-4 exhibits a similar magnitude of calcium response as that elicited by the ATP/glutamate cocktail, but pre-exposure to Ex-9 reduces the magnitude of this response (G). In contrast, the magnitude of the calcium response in neurons to Ex-4 was not further inhibited by Ex-9 (H). For (G) and (H), data shown as mean ± SEM. *, $p<0.05$.

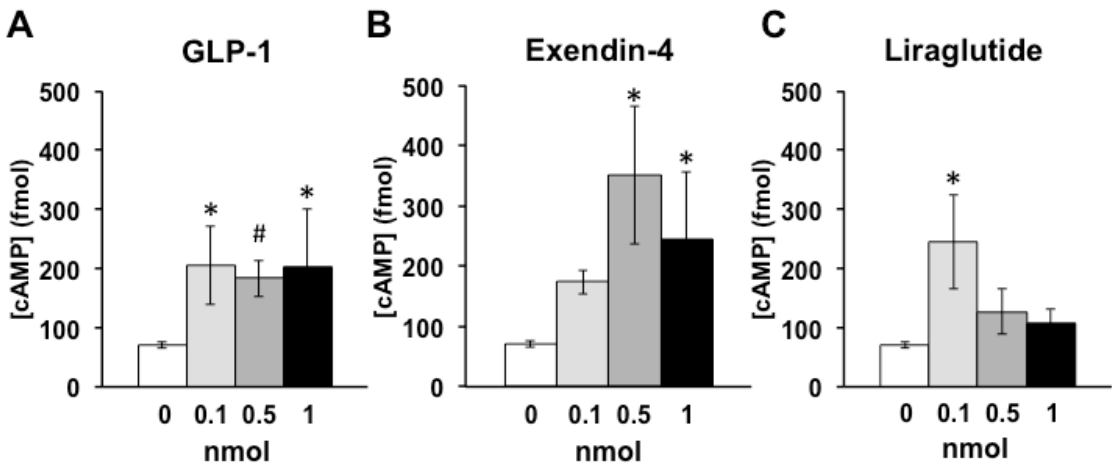

Figure 5. GLP-1R activation increases cAMP concentration in astrocytes *in vitro*. Bath application of the GLP-1R agonists GLP-1 (A), Ex-4 (B), or liraglutide (C) elevated cAMP levels in immortalized rat type-1 astrocytes (DI-TNC1). For each agonist/dose combination, n=3 plates of cells; for control, n=9 plates. Data shown as mean ± SEM. *, $p<0.05$; #, $p<0.08$ compared to control (dose 0).

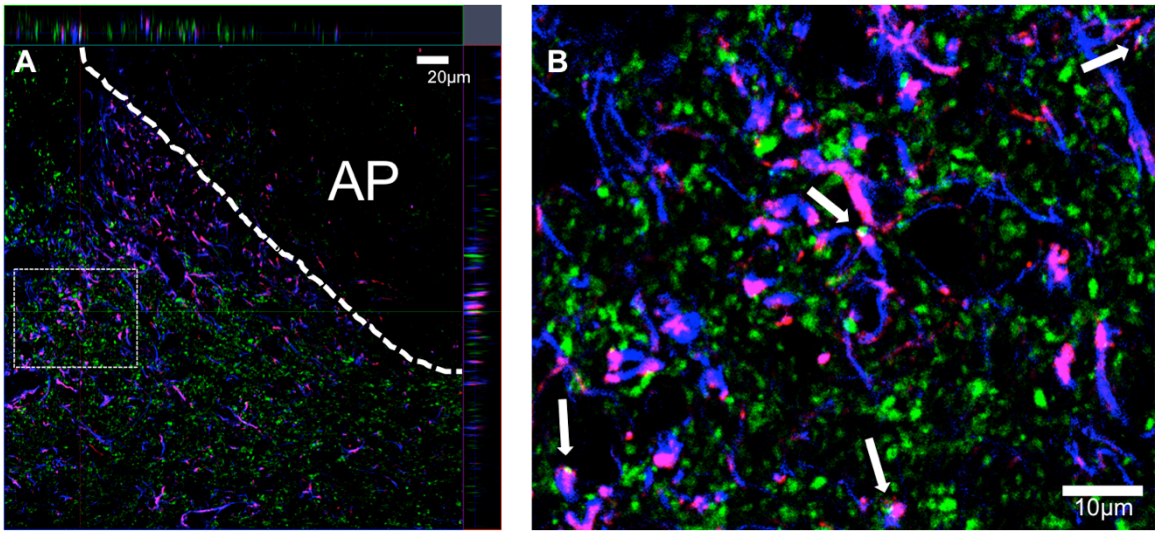

Figure 6. GLP-1-containing axons form close appositions with NTS astrocytes. Representative 1.5 μm thick images from NTS-containing transverse sections (n=3 rats) show GLP-1 in magenta, GFAP (astrocyte marker) in blue, and synaptophysin in green. White arrows indicate triple-labeling, providing evidence for synaptic-like contacts between GLP-1-containing cells and astrocytes in the NTS. The top orthogonal in (A) represents the XZ plane for the Z stack, whereas the right orthogonal represents the YZ plane for the Z stack. The cross hairs in this panel (red vertical line meets green horizontal line) highlight one of several examples of three-label evidence for appositions at this level of the NTS. The inset in (B) reveals this at higher resolution, with the three-label example at the cross hairs in (A) represented by the top arrow in (B). AP = area postrema.

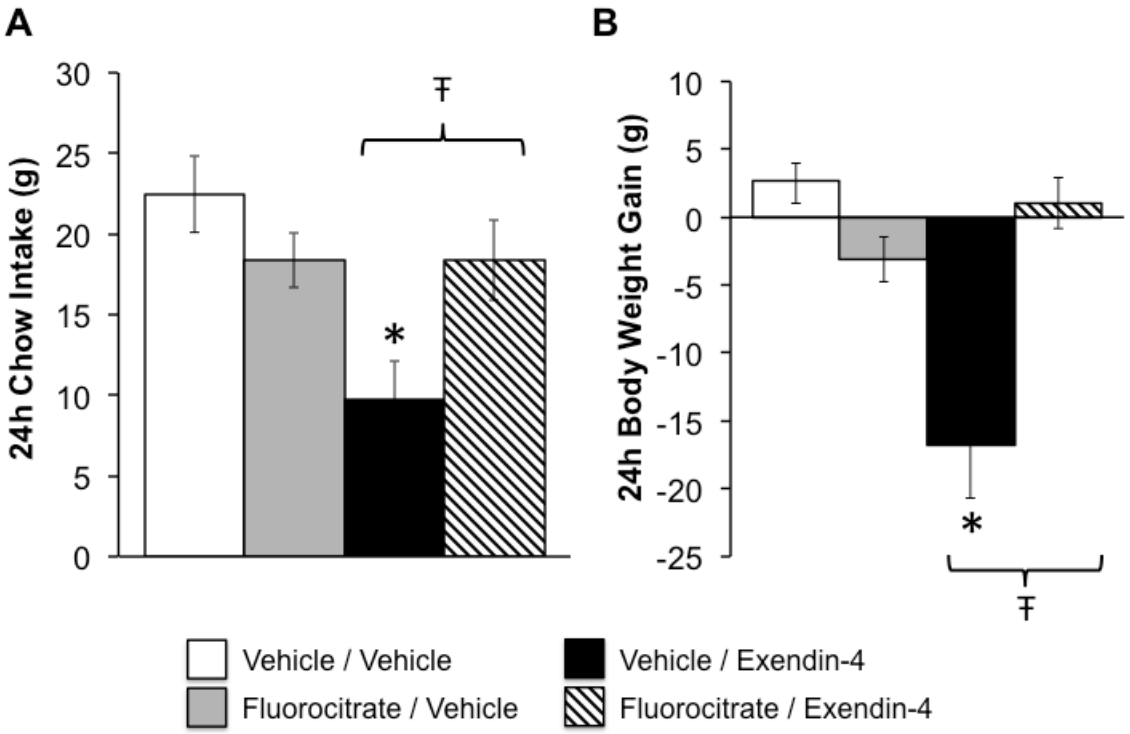

Figure 7. Inhibition of mNTS astrocytes attenuates the food intake- and body weight-suppressive effects of mNTS GLP-1R activation. Rats (n=5) were given an intra-mNTS pretreatment with the astrocyte Krebs cycle inhibitor fluorocitrate or its vehicle, followed by direct mNTS administration of the GLP-1R agonist Ex-4. Fluorocitrate, at a dose that on its own had no effect on chow intake or body weight gain, significantly attenuated the ability of intra-mNTS Ex-4 to reduce chow intake (A) and body weight gain (B) over the 24h post-injection. * significantly different from vehicle/vehicle ($p<0.05$); Ŧ $p<0.05$ versus vehicle/Ex-4. Key applies to both panels. Data shown as mean ± SEM.

CHAPTER 3: Glucagon-Like Peptide-1 Receptor Signaling In The Lateral Dorsal Tegmental Nucleus Regulates Energy Balance

David J. Reiner[1], Rosa Leon[1], Lauren E. McGrath[1], Kieran Koch-Laskowski[1], Joel D. Hahn[2], Scott E. Kanoski[3], Elizabeth Mietlicki-Baase[1], and Matthew R. Hayes[1]

[1]Translational Neuroscience Program, Department of Psychiatry, Perelman School of Medicine

[2]Department of Biological Sciences, Neurobiology Section, University of Southern California, Los Angeles, CA, 90089, USA

[3]Department of Biological Sciences, Human and Evolutionary Biology Section, University of Southern California, Los Angeles, CA, 90089, USA

Abstract

Glucagon-like peptide-1 (GLP-1) synthesized in the nucleus tractus solitarius (NTS) of the hindbrain is physiologically required for the normal control of food intake, while pharmacological GLP-1 receptor (GLP-1R) agonists are FDA-approved for the treatment of diabetes and obesity. However, the neurobiological substrates that mediate the anorectic effects of both endogenous GLP-1 and exogenous GLP-1R agonists require further investigation. As the lateral dorsal tegmental nucleus (LDTg) expresses the GLP-1R and represents a potential neuroanatomical hub connecting the NTS with the other nuclei in the midbrain and forebrain, we tested the hypothesis that GLP-1R signaling in the LDTg regulates food intake. Direct activation of LDTg GLP-1R suppresses food intake through a reduction in meal size and independent of nausea/malaise. Immunohistochemical data show that GLP-1-producing neurons in the NTS project to the LDTg, providing anatomical evidence of endogenous central GLP-1 in the LDTg. Pharmacological blockade of LDTg GLP-1Rs with exendin-(9-39) dose-dependently increases food intake and attenuates the hypophagic effects of gastric distension. As GLP-1 mimetics are administered systemically in humans, we evaluated whether peripherally administered GLP-1R agonists access the LDTg to affect feeding. Immunohistochemical data show that a systemically-administered fluorescent GLP-1R agonist accesses the LDTg and is juxtaposed with neurons. Additionally, blockade of LDTg GLP-1Rs attenuates the hypophagic effects of a systemic GLP-1R agonist. Together, these data indicate that LDTg GLP-1R signaling controls energy balance and underscores the role of the LDTg in integrating energy balance-relevant signals to modulate feeding.

Introduction

Among the many hormones, neuropeptides, and neurotransmitters that influence feeding behavior, the glucagon-like peptide-1 (GLP-1) system stands out as a key regulator of physiological and behavioral processes involved in glycemic and food intake control (Grill and Hayes, 2012). Within the central nervous system (CNS), GLP-1 is produced from the preproglucagon (PPG) neurons of the caudal nucleus tractus solitarius (NTS). These PPG neurons project widely throughout the CNS (Dossat et al., 2011; Alhadeff et al., 2012; Kanoski et al., 2016), suggesting that GLP-1 receptor (GLP-1R) populations that are distributed across the neuraxis (Merchenthaler et al., 1999) are activated by centrally-produced GLP-1, either through direct projections of PPG neurons or through volume transmission (Dossat et al., 2011; Alhadeff et al., 2012; Hsu et al., 2015; Kanoski et al., 2016). However, the functional role of each of these GLP-1R populations in mediating the intake suppressive effects of exogenous GLP-1R agonists and/or endogenous NTS-derived GLP-1 remains largely unresolved. This gap in our knowledge is of clinical significance, as FDA-approved GLP-1R agonists penetrate into the CNS to activate central GLP-1Rs to suppress food intake (Hayes et al., 2011a; Kanoski et al., 2016; Reiner et al., 2016).

The lateral dorsal tegmental nucleus (LDTg) of the mesopontine tegmentum expresses GLP-1Rs (Merchenthaler et al., 1999), receives axonal projections from the hindbrain, hypothalamus, and midbrain structures (Cornwall et al., 1990), and is anatomically positioned to serve as a potential hub modulating energy balance and motivated behavior (Reiner et al., 2017). Indeed, prior research has shown that other neuropeptide systems act in the LDTg to modulate energy balance, establishing a role for the LDTg in food intake and meal size control, body weight regulation, and appetitive

behavior (Dickson et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012; Reiner et al., 2017). The NTS projects to the LDTg, and critically, these projections originate at the same rostral-caudal level as NTS PPG neurons (Cornwall et al., 1990). This suggests that a portion of these LDTg-projecting NTS neurons is GLP-1-positive and may provide an endogenous central source of GLP-1 to the LDTg. As the NTS receives input from the gastrointestinal (GI) tract via the vagus nerve (Grill and Hayes, 2009, 2012), these anatomical findings provide the intriguing possibility of a potential role of gut-LDTg signaling through a NTS-to-LDTg GLP-1 pathway.

Given that the LDTg expresses GLP-1R and integrates information from peripherally and centrally-derived energy status signals to affect energy balance (Dickson et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012; Reiner et al., 2017), we examined the hypothesis that GLP-1R signaling in the LDTg is both pharmacologically and physiologically relevant for energy balance control. Current data show that LDTg GLP-1R activation decreases food intake through a reduction in meal size and endogenous NTS-to-LDTg GLP-1 signaling mediates the intake suppressive effects of a self-ingested mixed macronutrient preload. Complementary anatomical and behavioral data show that LDTg GLP-1Rs are a CNS site-of-action mediating the intake suppressive effects of systemic GLP-1R agonists. These data establish a novel role of GLP-1 signaling in the LDTg for energy balance control and provide further anatomical and behavioral evidence that the LDTg is a potential hub mediating ingestive behavior.

Materials and Methods

Animals

Male Sprague-Dawley rats (310-325g upon arrival; Charles River, Wilmington, MA, USA or Harlan/Envigo, Indianapolis, IN, USA) were individually housed in hanging wire cages (12h light/dark cycle) and given *ad libitum* chow (Purina LabDiet 5001, Quakertown, PA, USA) and water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and at the University of Southern California and were performed according to the National Institutes of Health guidelines.

Drugs

Exendin-4 (Ex-4; Bachem, Torrance, CA, USA) was dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA, USA) for central injections and 0.9% sterile saline for peripheral injections. Exendin-(9-39) (Ex-9; Bachem, Torrance, CA, USA) was dissolved in aCSF. Fluorescent exendin-4 (FLEX; Anaspec, Freemont, CA, USA) was dissolved in 99.97% saline and 0.03% aCSF.

Stereotaxic surgery

Animals were anesthetized with an intramuscular (IM) injection of a cocktail (KAX) composed of ketamine (90mg/kg), acepromazine (0.64mg/kg), and xylazine (2.7mg/kg) and placed in a stereotaxic apparatus. Bilateral guide cannulae (Plastics One, Roanoke, VA, USA) targeting the LDTg (according to Paxinos & Watson, 2004; coordinates: ±0.5mm lateral to midline, 8.7 mm posterior to bregma, 4.1 mm ventral to skull; microinjector aimed 6.6 mm ventral to skull) were implanted and affixed to the skull

with bone screws and dental cement. Analgesia (meloxicam, 2 mg/kg) was administered to all animals following surgery. Rats were allowed ~1 week recovery from surgery before the beginning of an experiment. LDTg injection placements were verified histologically postmortem by intraparenchymal injections of pontamine sky blue (100 nl). Animals with missed cannula placements were eliminated from analyses.

Immunohistochemistry

GLP-1 axon labeling in the LDTg

Immunohistochemical labeling of GLP-1 axons in the LDTg was performed as previously described (Hsu et al., 2015). Briefly, rats (n=4) were transcardially perfused and brains were removed and sectioned coronally at 30µm. LDTg-containing sections were incubated for 60h at 4°C in primary antibody for rabbit anti-GLP-1 (1:2000, Peninsula Labs, San Carlos, CA, USA) in potassium phosphate buffered saline (KPBS) with 0.1% Triton X-100. Following primary antibody incubation, sections were incubated overnight at 4°C with biotinylated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA, USA) in KPBS with 0.1% Triton X-100. Following a KPBS rinse, sections were incubated with KPBS with ABC reagent (1:1000, reagent A and B from ABC Elite kit, Vector Labs, Burlingame, CA, USA) for 4h. Following further KPBS washes, sections were reacted for 10 min in KPBS containing 0.005% H_2O_2 and 0.05% 3,3'-Diaminobenzidine. Following final washes, the sections were mounted on glass slides, air-dried, and coverslipped with DePeX. Photomicrographs were taken with a digital camera mounted to a microscope (Nikon 80i) equipped for darkfield illumination.

Retrograde tracing of GLP-1 NTS-to-LDTg projections

Animals were anesthetized with a surgical dose of KAX and received a unilateral LDTg injection of 100nl of 0.25% cholera toxin B subunit (CTB; #104, List Laboratories, Campbell, CA, USA) over 2s, according the following coordinates (according to Paxinos & Watson, 2004): 0.6mm lateral to midline, 8.7 mm posterior to bregma, 4.5 mm ventral to skull; microinjector aimed 7.0 mm ventral to skull. After the injection, the microinjector was left in place for ten minutes before being removed and the incision was sutured. Rats were perfused seven days later. Following removal, brains were postfixed in 4% paraformaldehyde for 6h and cryoprotected in 20% sucrose in 0.1M PBS at 4°C for 2-3 days. Coronal brain sections (30 μm) encompassing the NTS or LDTg were sliced and stored in cryoprotectant until processing. Sections were washed in 50% ethanol for 30 min, rinsed in PBS, and then incubated in 1% sodium borohydride for 20 min. Following a PBS wash, sections were then blocked in PBS with 5% normal donkey serum and 0.2%Triton-X at room temperature for 1h. NTS-containing sections were incubated overnight at room temperature in the following primary antibodies: mouse anti-CTB (1:1000, ab62429, Abcam, Cambridge, MA, USA) and rabbit anti-GLP-1 (1:1000, T-4363, Peninsula Labs, San Carlos, CA, USA). LDTg-containing sections were incubated overnight in the mouse anti-CTB antibody. Following a PBS rinse, sections were incubated for 2h in the appropriate donkey anti-primary antibody species Alexa Fluor 488 and 594 antibodies (Jackson Immunoresearch, West Grove, PA, USA). All antibody incubations were performed in blocking solution. Sections were mounted on slides and visualized with a Leica SP5 X confocal microscope using the 488 and 594 laser lines with a 20x objective. All images were collected sequentially to avoid contamination of

signals from other fluorophores. At least six NTS-containing sections from 14.2-14.7 mm posterior to bregma were used to quantify the number of CTB and PPG neurons.

Fluorescent exendin-4 labeling in the LDTg

To provide anatomical evidence that a systemically administered GLP-1R agonist penetrates into the brain and binds to cells within the LDTg, rats received an IP injection of fluorescent exendin-4 (FLEX; 3 µg/kg, n=5) at the onset of the dark cycle. Rats were food deprived for one hour prior to dark cycle onset and food remained unavailable. Three hours later, rats were anesthetized with an IM injection of the surgical dose of KAX and transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde in 0.1M PBS. Brains were removed, postfixed, sectioned, and tissue was blocked as described above. LDTg-containing sections were incubated overnight in primary antibodies [rabbit anti-glial fibrillary acidic protein (GFAP; 1:2000, Z0334, Dako/Agilent, Santa Clara, CA, USA) and mouse anti-NeuN (1:1000, MAB377, Millipore, Billerica, MA, USA)] in blocking solution. Following a PBS rinse, sections were incubated in appropriate donkey Alexa Fluor 594 and 647 secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) at room temperature. Sections were mounted on slides and visualized with a Leica SP5 X confocal microscope using the 488, 594, and 633 laser lines with a 63x oil immersion objective. FLEX was visualized in the LDTg using the 488 laser line. All images were collected sequentially to avoid contamination of signals from other fluorophores. Images are represented as a maximal z-stack projection with Fiji 2.0 or as a 3D rotational video with Imaris (8.4.1).

Behavioral testing

General Procedures

Drug injections were performed immediately prior to the onset of the dark cycle unless otherwise specified. For experiments measuring *ad libitum* food intake, food weights were measured at 1, 3, 6, and 24h after injection, unless otherwise noted. Food weights were recorded to the nearest 0.1g, accounting for spillage. Body weight was measured at 0 and 24h after injection. Treatments were assigned using a within-subject counterbalanced design, with at least 72h between injections.

Intra-LDTg exendin-4 dose-response experiments

To assess the dose-response effects of LDTg GLP-1R activation on food intake and body weight, rats (n=10) received unilateral LDTg injections of Ex-4 (0, 0.025, 0.05µg; 100 nl aCSF) immediately before the onset of the dark cycle. These doses of Ex-4 were selected based on previous dose-response experiments in the NTS, ventral tegmental area (VTA), and nucleus accumbens (NAc) (Hayes et al., 2008; Alhadeff et al., 2012). Food intake and body weight were measured.

To determine if nausea/malaise contributes to the intake suppression following GLP-1R activation in the LDTg, pica, the intake of non-nutritive substances and a well-established model of nausea/malaise (Mitchell et al., 1977; Takeda et al., 1993; Yamamoto et al., 2002; Andrews and Horn, 2006; Kanoski et al., 2012a), was measured. Rats (n=8) were given access to kaolin clay (Research Diets, New Brunswick, NJ, USA) for ~1 week prior to the experiment. Chow and kaolin clay intake were measured at 24h after unilateral LDTg injection of exendin-4 (0, 0.025, 0.05µg; 100 nl aCSF).

For meal pattern experiments, rats (n=10) were housed in a custom-made automated feedometer system consisting of hanging wire cages with access to a food cup on an electronic scale (Hayes et al., 2011b; Kanoski et al., 2012b; Mietlicki-Baase et al., 2013b), which records the weight of food cups every 10s through computer software (Labview). A meal was defined as at least 0.25g of food ingested with 10 minutes or more between feeding bouts (Hayes et al., 2011b; Kanoski et al., 2012b; Mietlicki-Baase et al., 2013b). Meal patterns were assessed at 12 and 24h post LDTg injection of exendin-4 (0, 0.025, 0.05μg; 100 nl aCSF).

LDTg GLP-1R blockade feeding experiments

To determine the physiological role of GLP-1R signaling in the LDTg for energy balance control, we assessed the dose response of GLP-1R blockade in the LDTg on food intake and body weight gain. Rats (n=9) received unilateral LDTg injections of the competitive GLP-1R antagonist Ex-9 [0, 10, 20μg; 200 nl aCSF; doses chosen based on (Hayes et al., 2009)] immediately before the onset of the dark cycle. Food intake and body weight were measured.

To examine whether GLP-1R blockade in the LDTg attenuates the intake-suppressive effects of voluntary ingestion of a nutritionally complete preload, rats (n=5) were trained to drink 9ml of vanilla Ensure® (Abbott Nutrition, Abbott Laboratories, Columbus, Ohio) in 15 min just prior to dark cycle onset. Immediately following the ingestion of Ensure®, rats received unilateral LDTg injections of Ex-9 (0, 10μg; 100 nl aCSF; dose chosen to be subthreshold for effect alone on feeding when administered in the LDTg) in a counter-balanced within subjects design. Injections were separated by at least 48h and following training, rats only received access to Ensure® on injection days.

Chow was removed during the 15min Ensure® access period and was given back immediately following the injection. Chow intake was measured at 0.5, 1, 1.5, 2, 4, and 24h after injection, and body weight was measured at 0 and 24h after injection.

To examine whether acute pharmacological blockade of LDTg GLP-1R attenuates the intake-suppressive effects of systemic Ex-4 administration, we injected separate rats (n=9) with Ex-9 (0, 10µg; 100 nl aCSF; dose chosen to be subthreshold for effect alone on feeding when administered in the LDTg) unilaterally in the LDTg 1h prior to the onset of the dark cycle. Fifteen minutes prior to the onset of the dark cycle, rats were injected systemically with Ex-4 (3 µg/kg, IP), and subsequent food intake was measured.

Statistical analyses

All data are represented as mean ± SEM with the α level set to p=0.05. Statistical analyses were performed using Statistica (Statsoft). For behavioral studies, binned data were analyzed using separate repeated measures one-way ANOVAs that accounted for the within-subjects experimental design. Statistically significant effects were probed using Student Neuman-Keuls *post-hoc* analyses or planned comparisons except when noted.

Results

LDTg GLP-1R activation reduces cumulative chow intake and body weight

As the LDTg expresses GLP-1R mRNA and receives projections from the NTS, a region that contains GLP-1-producing neurons (Cornwall et al., 1990; Merchenthaler et al., 1999), we first tested whether activation of GLP-1R in the LDTg suppresses food intake and body weight. Unilateral administration of Ex-4 in the LDTg decreases chow intake at 6 and 24h after injection compared to aCSF vehicle treatment ($F_{2,18} \geq 5.64$, $p<0.05$; Figure 1A). Post-hoc analyses reveal that both doses of Ex-4 significantly suppress chow intake at 6 and 24h post-injection ($p<0.05$ compared to vehicle). Intra-LDTg Ex-4 also significantly decreases 24h body weight change ($F_{2,18}=11.16$, $p<0.001$; Figure 1B), with post-hoc analyses showing a significant effect with either dose of Ex-4 ($p<0.05$ compared to vehicle). Importantly, behavioral data from animals (n=6) with missed LDTg cannula placements does not show hypophagia after GLP-1R activation (See Figure A2 in Appendix; $F_{1,3}<0.23$, $p>0.80$).

LDTg GLP-1R activation does not produce malaise

To determine if the intake suppression following LDTg GLP-1R activation is driven by nausea/malaise, we injected the same doses of Ex-4 into the LDTg and measured pica. Intra-LDTg significantly decreases 24h chow intake compared to vehicle ($F_{2,14}=3.91$, $p<0.05$; Figure 1C) with post-hoc analyses showing that the higher dose of Ex-4 significantly reduces chow intake ($p<0.05$). Importantly, intra-LDTg has no effect on 24h kaolin intake ($F_{2,14}=2.00$, $p<0.05$; Figure 1D), suggesting that nausea/malaise is not driving the hypophagia following intra-LDTg GLP-1R activation.

LDTg GLP-1R activation suppresses meal size

Meal pattern analyses show that unilateral injection of Ex-4 in the LDTg significantly decreases meal size at 12h and 24h ($F_{2,18}$>5.73, p<0.05; Figure 1E), but has no effect on meal number at either time point ($F_{2,18}$<0.08, p>0.9; Figure 1F). Post-hoc analyses reveal that both doses of Ex-4 suppress meal size at 12 and 24h post-injection (p<0.05). These data suggest that LDTg GLP-1R activation reduces food intake predominantly through a reduction in meal size with minimal effects on meal number.

GLP-1-expressing axon terminals are present in the LDTg, and NTS PPG neurons project to the LDTg

Central GLP-1 is produced by PPG neurons in the NTS, which project widely throughout the CNS, including to the VTA and NAc (Vrang et al., 2003; Alhadeff et al., 2012; Grill and Hayes, 2012; Hayes et al., 2014b; Kanoski et al., 2016). We therefore performed immunohistochemical analyses to test whether GLP-1 axons are also present in the LDTg. Indeed, GLP-1-immunopositive axons are present in the LDTg (Figure 2A-B).

To directly determine the endogenous source of GLP-1 axons projecting into the LDTg, the retrograde tracer CTB was injected into the LDTg (n=3) and animals were sacrificed seven days later. Retrograde labeling of CTB shows that 4.9±1.0% of CTB-immunopositive NTS neurons colocalize with PPG and 4.8±1.7% of PPG-immunopositive neurons colocalize with CTB (see Figure 2C-E for representative images). A representative image shows that the CTB injection site from this same animal is within the LDTg (Figure 2F). These data provide evidence that a portion of NTS PPG neurons project to the LDTg. Collectively, IHC data showing the presence of GLP-1-

immunopositive fibers in the LDTg and that NTS PPG neurons project to the LDTg provide anatomical evidence of a physiological role of endogenous GLP-1R signaling in the LDTg.

LDTg GLP-1R blockade increases food intake

Intra-LDTg GLP-1R blockade with the competitive GLP-1R antagonist Ex-9 significantly increases food intake ($F_{2,16}$=3.64, p<0.05; Figure 3A) at 24h with no effect on body weight ($F_{2,16}$=0.19, p=0.8; Figure 3B). Importantly, the 10µg dose of Ex-9 has no effect on food intake compared to aCSF vehicle (p>0.05), but the 20µg dose of Ex-9 significantly increases food intake at 24h (p<0.05). These data suggest that GLP-1R signaling in the LDTg is physiologically relevant for food intake control.

LDTg GLP-1R blockade attenuates the intake suppressive effects of a self-ingested preload

To determine the potential role of a gut-to-NTS-to-LDTg GLP-1 pathway in mediating food intake, we next examined if LDTg GLP-1R blockade can attenuate the intake suppressive effects of a voluntary ingested gastric preload (9 ml Ensure®) gastric distension. To avoid competing orexigenic and anorectic behavioral responses, we intentionally used a dose of Ex-9 that is subthreshold for an effect on feeding when delivered unilaterally in the LDTg. GLP-1R blockade in the LDTg significantly attenuates the intake suppressive effects of gastric distension at 2 and 24h post-injection ($F_{1,4}$>8.76, p<0.05 Figure 3C) and approaches significance at 4h ($F_{1,4}$=5.98, p=0.07). Intra-LDTg Ex-9 also significantly increases body weight compared to aCSF vehicle treatment ($F_{1,4}$=9.53, p<0.05; Figure 3D). These data show that LDTg GLP-1R blockade can

attenuate the intake suppressive effects of gastric distension, suggesting physiological relevance of either direct or indirect gut-to-LDTg signaling.

Peripherally-administered fluorescent exendin-4 is juxtaposed with LDTg neurons

Given that GLP-1 mimetics are administered systemically in humans, we next evaluated whether a peripherally-administered fluorescent GLP-1R agonist accesses the LDTg of rats. Indeed, systemically-administered FLEX shows juxtaposition with neurons but minimal association with astrocytes in the LDTg (Figure 4A-C).

LDTg GLP-1R blockade attenuates the intake suppressive effects of a systemically-administered GLP-1R agonist

Given anatomical data showing that a systemically-administered GLP-1R agonist accesses the LDTg, we next evaluated whether LDTg GLP-1R blockade attenuates the anorectic effects of systemic Ex-4. Systemic injection of Ex-4 significantly suppresses chow intake at 3h (main effect of Ex-4, $F_{1,8}$=6.31, $p<0.05$) and approaches significance at 6h (main effect of Ex-4, $F_{1,8}$=5.15, $p=0.053$) after injection (Figure 4C). There is also a significant interaction between Ex-4 and Ex-9 at 3 and 6h after injection ($F_{1,8}\geq6.44$, $p<0.05$); post-hoc analyses show that pre-treatment with intra-LDTg Ex-9 reverses the intake-suppressive effects of peripheral Ex-4 at 3h and 6h post-injection ($p<0.05$). Importantly, LDTg GLP-1R blockade alone (Ex-9/vehicle) does not significantly increase chow intake at any time point (all $p>0.05$ compared to vehicle/vehicle), though there is a significant main effect of Ex-9 at 3 and 6h ($F_{1,8}\geq5.27$, $p<0.05$) driven by the Ex-9/Ex-4 condition. These data show that intra-LDTg GLP-1R blockade attenuates the hypophagic effects of systemically administered Ex-4, suggesting the potential pre-clinical relevance of LDTg GLP-1R signaling. Collectively, these behavioral data

complement the anatomical data in Figure 4A-B and highlight that peripherally administered Ex-4 accesses the LDTg in a functional capacity, suggesting the pre-clinical relevance of GLP-1R signaling in the LDTg for energy balance control.

Discussion

To improve current obesity pharmacotherapies, research aimed at understanding neuroendocrine signals and the neurobiological substrates that control energy balance is required. Recently, much attention has been focused on the GLP-1 system, as GLP-1 mimetics are FDA approved for diabetes and obesity treatment (Apovian et al., 2015; Kanoski et al., 2016). GLP-1R are widely expressed throughout the CNS, and central endogenous GLP-1, as well as exogenous long-lasting GLP-1R agonists activate these central GLP-1Rs, ultimately leading to reduced food intake and body weight (Kanoski et al., 2016). Given that GLP-1R are expressed widely throughout the CNS (Merchenthaler et al., 1999), it is important to examine the specific brain regions that mediate the effects of GLP-1R activation. The LDTg of the mesopontine tegmentum expresses GLP-1R (Merchenthaler et al., 1999), and has been shown to be a relevant nucleus for energy balance control and motivated behaviors (Schmidt et al., 2009; Dickson et al., 2010; Dickson et al., 2011; Lammel et al., 2012; Reiner et al., 2017). Here, we identify novel pharmacological and physiological roles of GLP-1R signaling in the LDTg for energy balance control.

Our data show that pharmacological activation of LDTg GLP-1Rs with direct intra-LDTg injection of the GLP-1R agonist Ex-4 decreases food intake and body weight, primarily through a reduction in meal size. These data are consistent with previous reports of central GLP-1R activation on energy balance that show similar magnitudes of intake suppression driven by reductions in meal size (Scott and Moran, 2007; Hayes et al., 2008; Grill and Hayes, 2009, 2012; Dossat et al., 2013; Hsu et al., 2015; Terrill et al., 2016). Though peripheral administration of GLP-1 mimetics produces nausea/malaise (Kanoski et al., 2012a), our data show that direct LDTg GLP-1R activation does not

produce pica, suggesting an absence of nausea/malaise following activation of this population of central GLP-1Rs. Collectively, these behavioral data expand the known relevant CNS sites of action for GLP-1 signaling in mediating energy balance.

The current studies also support a physiological role for LDTg GLP-1R signaling in energy balance control. Acute blockade of LDTg GLP-1R increases food intake, and at a dose subthreshold for an effect on feeding, blockade of GLP-1R in the LDTg can attenuate the intake suppressive effects of a mixed macronutrient preload. Collectively, these data provide novel evidence of a physiological role for LDTg GLP-1R signaling in energy balance control and suggest that the LDTg receives communication from the gut either through a humoral mechanism or via a vagal-to-NTS-to-LDTg pathway. Given that gastric distension activates PPG neurons in the NTS (Vrang et al., 2003; Hayes et al., 2009), combined with current data here showing that NTS PPG neurons project to the LDTg, the collective set of data would support the hypothesis that LDTg GLP-1 signaling mediates, at least in part, the satiation signaling arising from meal ingestion via a vagal-NTS-LDTg pathway. Indeed, IHC data show that GLP-1-immunopositive axons are present in the LDTg and that NTS PPG neurons project to the LDTg, providing anatomical evidence that NTS PPG neurons project to the LDTg and likely release GLP-1. Collectively, these data support a potential role of a gut-to-NTS-to-LDTg GLP-1 pathway that mediates energy balance control. These data underscore that the LDTg is a critical site of action for endogenous GLP-1R signaling under physiological conditions.

As FDA-approved GLP-1 mimetics are given systemically, it is important to examine the CNS nuclei that are activated by these peripherally administered GLP-1R agonists. Behavioral data suggest that systemically delivered GLP-1R agonists access

the LDTg, as LDTg GLP-1R blockade attenuates the intake suppressive effects of peripherally administered Ex-4. Immunohistochemical data provide further evidence that systemically-delivered GLP-1R agonists access the LDTg. Given the lack of a commercially available GLP-1R-specific antibody, we used peripheral administration of fluorescently-labeled Ex-4 to label GLP-1R-expressing cells (Reiner et al., 2016; Terrill et al., 2016). These data show that a systemically-delivered GLP-1 mimetic resistant to enzymatic degradation can access the LDTg, highlighting the potential pre-clinical relevance of the GLP-1R LDTg system. Interestingly, in contrast with our previous study showing FLEX localized to and internalized in both neurons and astrocytes within the NTS after IP administration (Reiner et al., 2016), FLEX associates predominately with neurons in the LDTg but only very minimally with LDTg astrocytes. These data indicate that GLP-1R may be expressed on different cell types depending on the CNS nucleus. Future investigation should examine the differential pharmacokinetics and receptor-binding rates throughout GLP-1R-expressing nuclei and how these cell types/regions might modulate energy balance, nausea, glycemic control, and motivated behaviors.

Collectively, our data show that LDTg GLP-1R signaling is pharmacologically and physiologically relevant for energy balance control and provide novel evidence of gut-NTS-LDTg GLP-1 signaling. Given that the LDTg is anatomically connected to feeding-relevant nuclei in the hindbrain, midbrain, hypothalamus, and forebrain (Cornwall et al., 1990), the current set of findings highlight the importance of investigating the LDTg and its role in integrating information from a wide range of CNS regions in order to mediate energy balance. Future studies should also explore how GLP-1 signaling in the LDTg interacts with other energy balance relevant signals to control energy balance as well as motivated behavior. Importantly, behavioral and anatomical data show that systemically

delivered long-acting GLP-1 mimetics can access the LDTg and regulate food intake, suggesting that the LDTg is of potential pre-clinical relevance for GLP-1-mediated energy balance control.

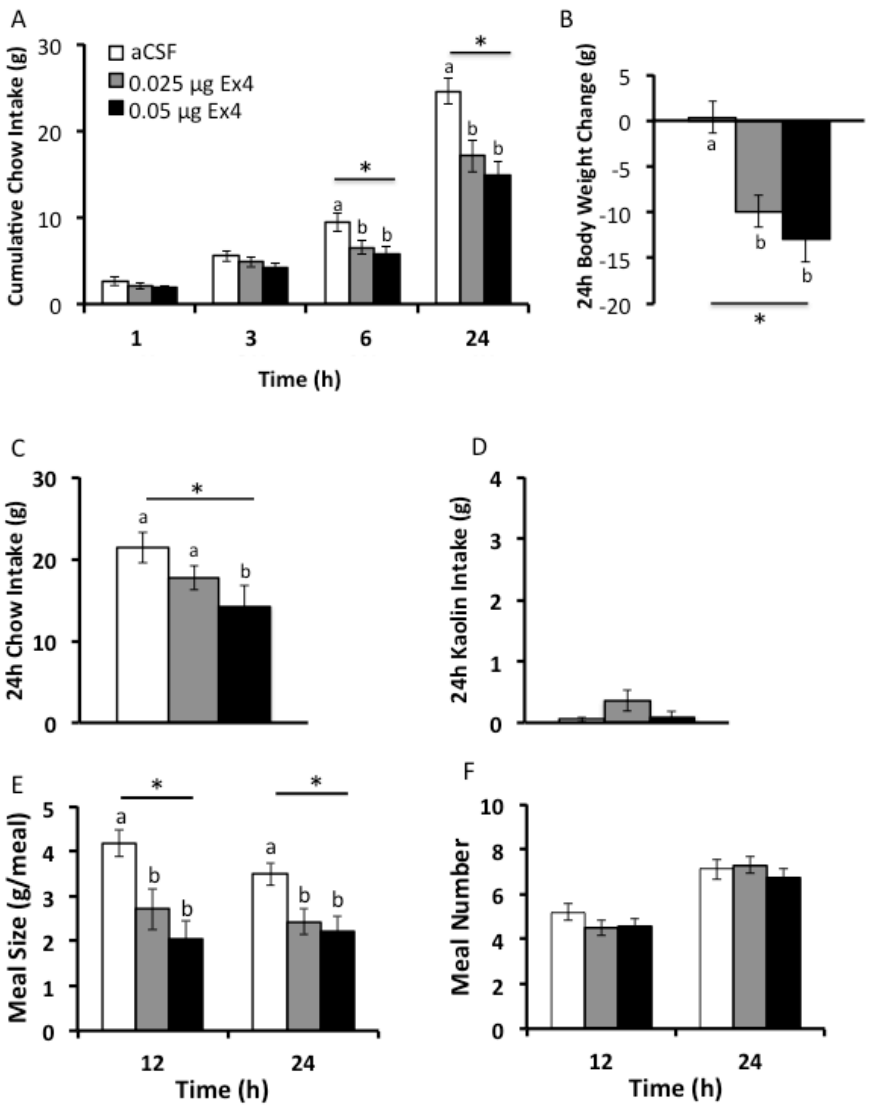

Figure 1. Intra-LDTg GLP-1R activation suppresses food intake through a decrease in meal size and independent of nausea. The GLP-1R agonist Ex-4 was unilaterally injected in the LDTg in a counterbalanced within-subjects design (n=10) at the onset of the dark cycle using the following doses: 0 (aCSF), 0.025, or 0.05µg. These doses of Ex-4 suppress food intake at 6 and 24h (A) and also reduce 24h body weight change (B). To determine if LDTg GLP-1R activation produces nausea/malaise, kaolin intake was measured following unilateral Ex-4 LDTg administration. A separate cohort of animals (n=8) received access to both chow and kaolin clay for one week prior to the beginning of the experiment. Intra-LDTg Ex-4 decreases 24h food intake (C) but has no effect on 24h kaolin intake (D). Meal patterns were analyzed in a third cohort of animals (n=10). Ex-4 decreases 12 and 24h meal size (E) but had no effect on meal number (F). * indicates significance by repeated measures ANOVA ($p<0.05$). Different letters are significantly different from each other ($p<0.05$) according to post-hoc tests. The key in (A) applies to all panels.

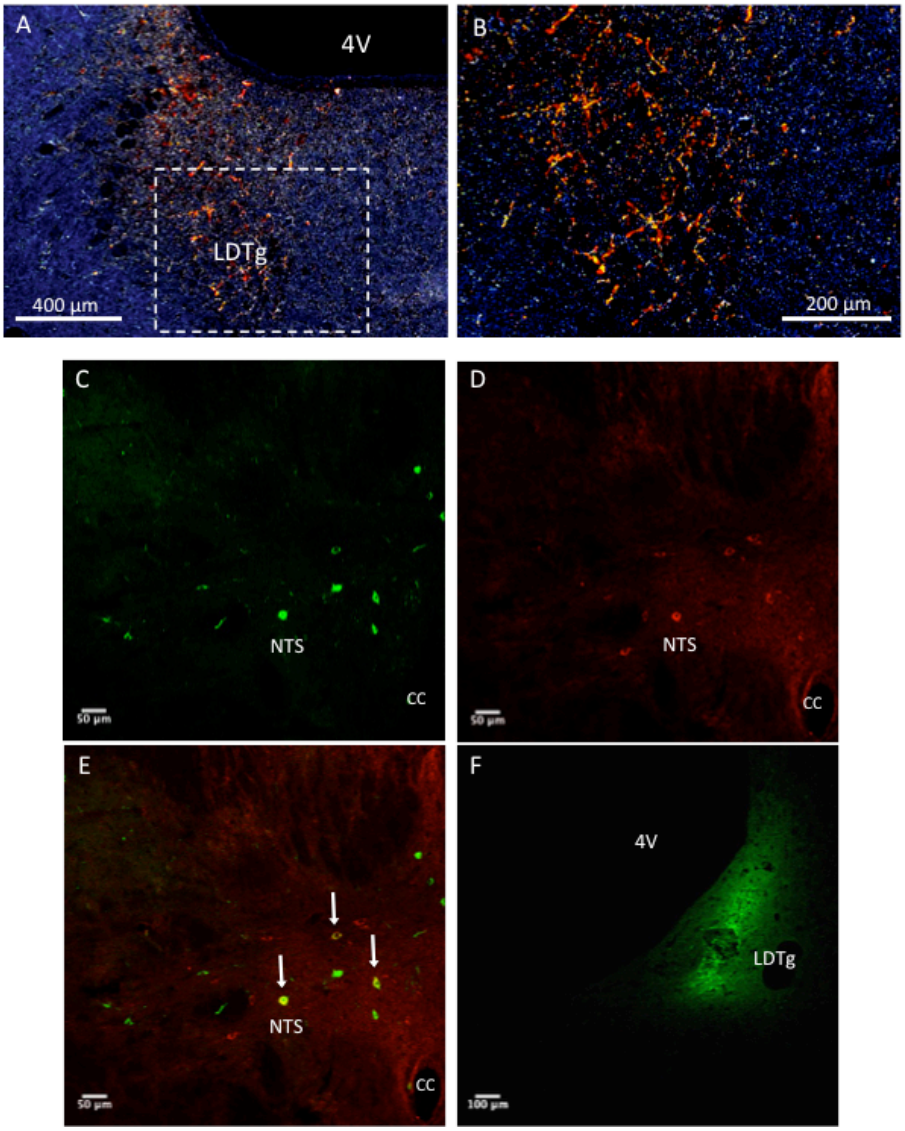

Figure 2. GLP-1-positive axons are present in the LDTg, and PPG neurons project from the NTS to the LDTg. Immunolabeling shows the presence of GLP-1-positive axons in the LDTg (n=4). Representative images are shown in (A-B). The white dashed-line box in A indicates the field of view in B. To determine if the NTS PPG neurons project to the LDTg, rats (n=3) were unilaterally injected with the neural pathway tracer CTB in the LDTg. Seven days later, rats were perfused and their brains were processed. Within the NTS, 4.9±1.0% of retrogradely-labeled CTB-positive neurons (green) co-localize with PPG-positive neurons (red). Representative images show CTB-positive neurons in green (C), PPG-positive neurons in red (D), and merged in (E). Figure 4F shows a representative CTB LDTg injection site. The white arrows in E indicate co-localization (yellow). 4V = 4th ventricle, cc= central canal, LDTg = lateral dorsal tegmental nucleus, NTS = nucleus tractus solitarius.

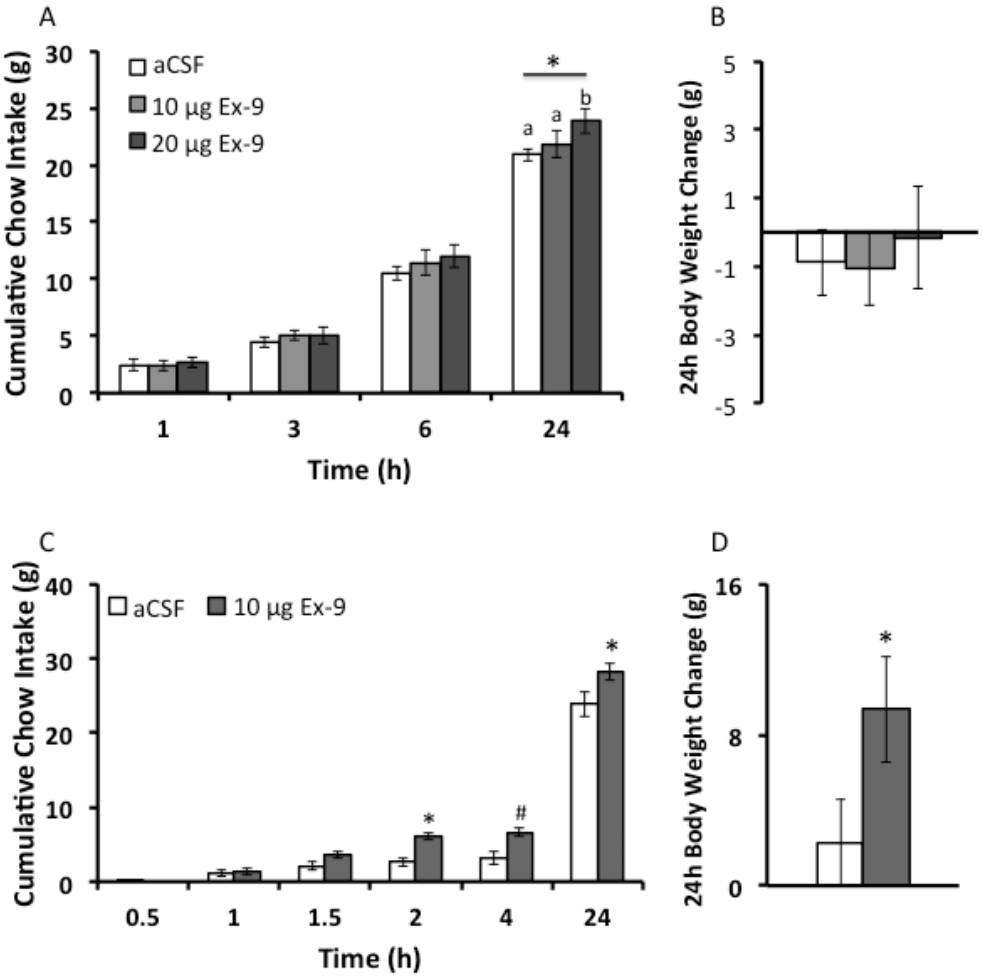

Figure 3: LDTg GLP-1R blockade increases food intake and attenuates the intake suppressive effects of a self-ingested preload. To determine the effect of GLP-1R blockade in the LDTg on food intake, the competitive GLP-1R antagonist Ex-9 was injected unilaterally in the LDTg in a counterbalanced within-subjects design at the onset of the dark cycle using the following doses: 0 (aCSF), 10, 20µg; 200nl (n=9). Only the highest dose of Ex-9 (20µg) increases food intake at 24h (A) and neither dose of Ex-9 had an effect on 24h body weight change (B). To determine if LDTg GLP-1R blockade can attenuate the hypophagic effects of gastric distension, rats (n=5) were trained to drink 9ml of Ensure® in fifteen minutes just prior to dark cycle onset. Following stable intake of Ensure®, rats received unilateral LDTg injections of Ex-9 [0 (aCSF), 10µg; 100 nl aCSF] immediately after the fifteen-minute Ensure® access period in a within-subjects design. Ex-9 significantly increases food intake at 2 and 24h after injection (C) and body weight over 24h (D). * indicates significance ($p<0.05$) and # indicates a trend for significance ($p=0.07$) by repeated measures ANOVA. Different letters are significantly different from each other ($p<0.05$) according to post-hoc tests. The key in (A) applies to (B), and the key in (C) also applies to (D).

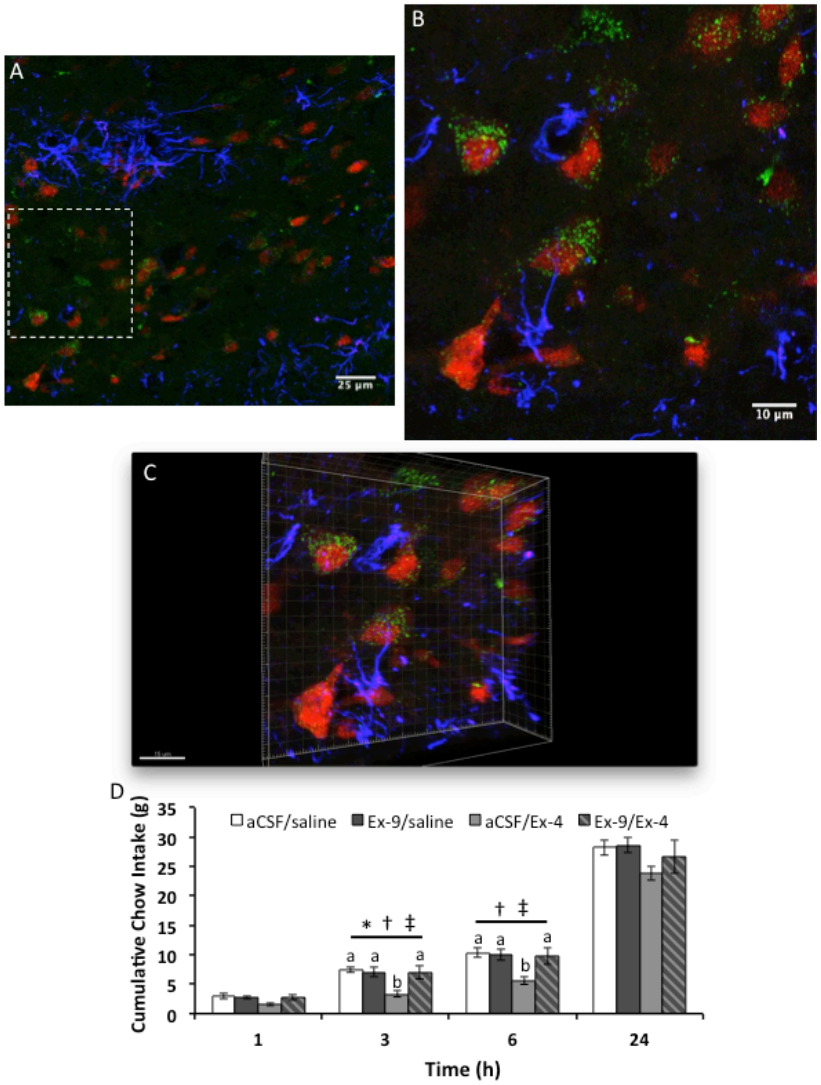

Figure 4: Systemically-delivered GLP-1R agonists access the LDTg. To determine if peripherally administered Ex-4 penetrates the LDTg, we injected fluorescently-tagged Ex-4 (FLEX, 3 µg/kg, IP), perfused the rats (n=5) 3h later, and processed the LDTg to visualize neurons, astrocytes, and FLEX. Peripherally administered FLEX (green) is juxtaposed with neurons (red) but minimally with astrocytes (blue) within the LDTg (A-B). 63x image in A, and 3x optical zoom of 63x in B. Dotted rectangle in A indicates field of view in B and C (still from 3D rotational video). To determine if GLP-1R blockade in the LDTg attenuates the hypophagic effects of peripheral Ex-4, the competitive GLP-1R antagonist Ex-9 was unilaterally injected in the LDTg (n=9) at a dose subthreshold for an effect on feeding (10µg; vehicle, 100nl aCSF) approximately 1h prior to the onset of the dark cycle. Fifteen minutes prior to the onset of the dark cycle, rats were injected systemically with Ex-4 [0 (saline), 3 µg/kg]. Ex-4 significantly suppresses food intake at 3h ($p<0.05$) and approaches significance at 6h post-injection ($p=0.06$), and pre-treatment with Ex-9 reverses this intake suppression (D). * indicates significant main effect of Ex-4 significance ($p<0.05$). † indicates a significant main effect of Ex-9 ($p<0.05$). ‡ indicates a significant interaction between Ex-4 and Ex-9 ($p<0.05$). Different letters are significantly different from each other ($p<0.05$) according to post-hoc tests.

Chapter 4: Amylin Acts In The Lateral Dorsal Tegmental Nucleus To Regulate Energy Balance Through GABA Signaling

David J. Reiner[1], Elizabeth G. Mietlicki-Baase[1], Diana R. Olivos[1], Lauren E. McGrath[1], Derek J. Zimmer[1], Kieran Koch-Laskowski[1], Joanna Krawczyk[1], Christopher Turner[1,2], Emily E. Noble[3], Joel D. Hahn[4], Heath D. Schmidt[2], Scott E. Kanoski[3], and Matthew R. Hayes[1]

[1]Translational Neuroscience Program, Department of Psychiatry, Perelman School of Medicine

[2]Department of Biobehavioral Health Sciences, School of Nursing, University of Pennsylvania

[3]Department of Biological Sciences, Human and Evolutionary Biology Section, University of Southern California, Los Angeles, CA, 90089, USA

[4]Department of Biological Sciences, Neurobiology Section, University of Southern California, Los Angeles, CA, 90089, USA

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Abstract

The pancreatic- and brain-derived hormone amylin promotes negative energy balance and is receiving increasing attention as a promising obesity therapeutic. However, the neurobiological substrates mediating amylin's effects are not fully characterized. We postulated that amylin acts in the lateral dorsal tegmental nucleus (LDTg), an understudied neural-processing hub for reward and homeostatic feeding signals. We used immunohistochemical (IHC) and quantitative PCR analyses to examine expression of the amylin receptor complex in rat LDTg tissue. Behavioral experiments were performed to examine the mechanisms underlying the hypophagic effects of amylin receptor activation in the LDTg. IHC and quantitative PCR analyses show expression of the amylin receptor complex in the LDTg. Activation of LDTg amylin receptors by the agonist salmon calcitonin dose-dependently reduces body weight, food intake, and motivated feeding behaviors. Acute pharmacological studies and longer-term adeno-associated viral (AAV)-knockdown experiments indicate that LDTg amylin receptor signaling is physiologically and potentially pre-clinically relevant for energy balance control. Finally, IHC data indicate that LDTg amylin receptors are expressed on gamma-aminobutyric acid (GABA)-ergic neurons and behavioral results suggest that local GABA receptor signaling mediates the hypophagia following LDTg amylin receptor activation. These findings identify the LDTg as a novel nucleus with therapeutic potential in mediating amylin's effects on energy balance through GABA receptor signaling.

Introduction

In the search for effective pharmacological treatments for obesity, much attention has focused on neuroanatomical targets in the central nervous system (CNS) such as the hypothalamus and caudal brainstem, each historically linked with the homeostatic regulation of energy balance (Grill, 2006; Lutz, 2006; Moran, 2009; Moran and Ladenheim, 2011; Grill and Hayes, 2012; Merlino et al., 2014). While these studies have informed the field about cellular and molecular mechanisms mediating the metabolic effects of many gastrointestinal- and adipose tissue-derived hormones, the chronic hyperphagia underlying human obesity does not appear to be related to disproportionate homeostatic feeding, but rather is more likely based on excessive appetitive and motivational processes directed towards the consumption of highly palatable/rewarding food (Lutter and Nestler, 2009; Halford et al., 2010; Kenny, 2011; Berthoud, 2012). Indeed, targeting non-homeostatic/reward-based systems may provide a unique opportunity to treat obesity and metabolic diseases (Narayanan et al., 2010; DiLeone et al., 2012). Urgently needed, however, is a deeper understanding of the relevant CNS reward circuitry and how it responds to and integrates energy balance signals to control food intake and body weight.

The lateral dorsal tegmental nucleus (LDTg) is a nucleus in the mesopontine tegmentum that is uniquely positioned as a processing hub for the integration of reward-based and homeostatic energy balance signaling (Cornwall et al., 1990; Schmidt et al., 2009; Dickson et al., 2011), yet has been understudied for its role in feeding and other motivational processes. Indeed, the LDTg has reciprocal projections with many feeding-relevant nuclei throughout the neuraxis, including but not limited to, the nucleus tractus solitarius (NTS), the ventral tegmental area (VTA), the lateral hypothalamus (LH), and

the parabrachial nucleus (PBN) (Cornwall et al., 1990). Given that the LDTg expresses receptors for a variety of feeding peptides (e.g. amylin, glucagon-like peptide-1, ghrelin, oxytocin, orexin and PYY) (Sexton et al., 1994; Merchenthaler et al., 1999; Parker and Herzog, 1999; Greco and Shiromani, 2001; Gould and Zingg, 2003; Dickson et al., 2011; Cabral et al., 2013), we hypothesize that energy balance-relevant neuroendocrine signals may act directly in the LDTg to modulate the neural processing of feeding-relevant information and affect motivational aspects of food reward.

Following initiation of a meal, a cascade of endocrine events occurs, including secretion of the peptide hormone amylin from the pancreatic β cells. Amylin activates its receptors within the CNS to suppress ongoing feeding during the meal and increase satiation (Roth et al., 2009; Lutz, 2012). Historically, the contribution of central amylin signaling to food intake control has centered on its action in homeostatic feeding centers, primarily the area postrema (AP) of the caudal brainstem (Lutz et al., 1998b; Lutz et al., 2001; Lutz, 2010b, a; Potes and Lutz, 2010; Potes et al., 2010; Potes et al., 2012; Roth et al., 2012), and secondarily in hypothalamic subnuclei including the arcuate nucleus (ARH) and ventromedial hypothalamus (VMH) (Trevaskis et al., 2010; Turek et al., 2010; Dunn-Meynell et al., 2016). However, recent work has also established the VTA and nucleus accumbens (NAc) as relevant nuclei for amylin's energy balance effects, particularly for reward-based feeding (Mietlicki-Baase et al., 2013b; Baisley and Baldo, 2014; Mietlicki-Baase et al., 2015b). While this growing body of literature highlights a more distributed CNS system mediating amylin's energy balance effects than originally thought, the action of amylin in these aforementioned nuclei cannot wholly explain the energy balance and food reward effects of amylin signaling (Lutz et al., 1998b; Mack et al., 2007; Mack et al., 2010). In fact, as the neural control of energy

balance is distributed across the CNS (Grill and Kaplan, 2002; Grill and Hayes, 2012) and *in vitro* radiography studies show that amylin binds to sites throughout the brain (Sexton et al., 1994; Becskei et al., 2004), the ability of amylin receptor signaling in other CNS nuclei to produce hypophagia requires more extensive evaluation.

Given that amylin is currently being considered as an anti-obesity therapeutic, it is especially critical to more fully understand the neural substrates mediating amylin's effects on reward-based feeding in addition to its impact on homeostatic intake (Lutz, 2012; Roth, 2013; Sadry and Drucker, 2013; Mietlicki-Baase and Hayes, 2014; Hay et al., 2015). That the LDTg binds amylin (Sexton et al., 1994) and is widely connected with a variety of energy balance-relevant nuclei (Cornwall et al., 1990), collectively supports our hypothesis that amylin receptor signaling in the LDTg may control food intake, body weight, and motivated behaviors directed towards food reward. Thus, data presented here lend greater insight into amylin receptor signaling through the CNS by identifying the LDTg as a novel nucleus mediating the anorexigenic effects of amylin, while underscoring the LDTg GABAergic system as a potential target for amylin-based therapies for the treatment of obesity.

Materials and Methods

Animals

Male Sprague-Dawley rats (310-325g upon arrival; Charles River, Wilmington, MA, USA) were individually housed in hanging wire cages (12h light/dark cycle) and had *ad libitum* access to chow (Purina LabDiet 5001, Quakertown, PA, USA) and water unless otherwise noted. For experiments labeling GABAergic neurons, male Sprague-Dawley rats (250g upon arrival; Envigo Labs, Indianapolis, IN, USA) were individually housed in hanging wire cages (12h light/dark cycle) and had *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at University of Pennsylvania or University of Southern California and were performed according to the National Institutes of Health guidelines.

Drugs

The amylin receptor agonist salmon calcitonin (sCT; Bachem, Torrance, CA, USA) was dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA, USA) for central injections or 0.9% sterile saline for peripheral injections. Amylin (Bachem Torrance, CA, USA), the amylin receptor antagonist AC187 (R&D Systems, Minneapolis, MN, USA), and saclofen (Sigma Aldrich, St. Louis, MO, USA) were dissolved in aCSF. Bicuculline (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 50% DMSO in aCSF-0.1M NaOH.

Stereotaxic surgery

Animals were anesthetized with a cocktail (KAX) composed of ketamine (90mg/kg), acepromazine (0.64mg/kg), and xylazine (2.7mg/kg; IM), and placed into a stereotaxic apparatus. Bilateral guide cannulae (Plastics One, Roanoke, VA, USA) targeting the LDTg (coordinates: ±0.5mm lateral to midline, 8.7 mm posterior to bregma,

4.1 mm ventral to skull; injector aimed 6.6 mm ventral to skull) were implanted and affixed to the skull with bone screws and dental cement. Analgesia was provided for all surgeries (meloxicam, 2 mg/kg). Rats were allowed ~1 week recovery from surgery before the beginning of an experiment. LDTg injection placements were verified histologically postmortem by either visualization of green fluorescent protein (GFP) in the calcitonin receptor (CTR) knockdown experiment or by intraparenchymal injections of pontamine sky blue (100 nl) in all other behavioral experiments. Animals with missed cannula placements were eliminated from analyses.

For lateral ventricle cannulae placements, rats were deeply anesthetized with a KAX cocktail composed of ketamine (90 mg/kg), acepromazine (0.72 mg/kg), and xylazine (2.8 mg/kg; IM), and placed in a stereotaxic apparatus. A stainless steel guide cannula (26-gauge Plastics One, Roanoke, VA) was surgically implanted toward the lateral ventricle using the following coordinates: 1.8mm lateral to bregma, 0.9 mm posterior to bregma, and 2.6 mm ventral from the surface of the skull, injector aimed 4.6 mm ventral from the skull. Following one week of recovery, placement of the cannula was verified by the elevation of cytoglucopenia resulting from a single injection of 2 µl of 105µg/µl 5-thio-D-glucose (Ritter et al., 1981). Animals that did not have at least 100% elevation of baseline blood glucose by 2 hours post injection were not used for colchicine injections.

Quantitative PCR

Chow-maintained rats (n=6) were euthanized 1-2h into the dark phase. Brains were rapidly removed, flash-frozen in −70 °C isopentane, and stored at −80 °C until processing. To examine the relative expression of the components of amylin receptor

complex in the LDTg via quantitative real-time PCR (qPCR), bilateral 1 mm^3 micropunches/hemisphere of LDTg-enriched tissue (approximately 8.2-9.2 mm posterior to bregma) were collected from each brain. Tissue samples were processed for the gene expression of components of the amylin receptor, the CTR and receptor activity modifying protein (RAMP) as previously described (Mietlicki-Baase et al., 2013b). Total RNA was extracted from the tissue samples using TRIzol (Invitrogen, Grand Island, NY, USA) and the RNeasy kits (Qiagen, Valencia, CA, USA) and used to synthesize cDNA with the Advantage RT-for-PCR kit (Clontech, Mountain View, CA, USA). Relative mRNA expression of each CTR and RAMP subtype was quantified using Taqman gene expression kits (CTR-A: Rn01526770_m1, CTR-B: Rn01526768_m1, RAMP1: Rn01427056_m1, RAMP2: Rn00571815_m1, RAMP3: Rn00824652_g1, GapDH: Rn01775763_g1) and PCR reagents from Applied Biosystems (Grand Island, NY, USA). Samples were analyzed with the Eppendorf Mastercycler ep realplex2. Relative mRNA expression calculations were completed using the comparative threshold cycle method (Bence et al., 2006; Mietlicki-Baase et al., 2013b). One sample was eliminated due to a technical error during RNA isolation.

Immunohistochemical analyses

Rats were deeply anesthetized with an IM injection of the surgical dose of KAX used for the LDTg cannulation surgeries and transcardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed, postfixed in 4% paraformaldehyde for 6h and subsequently cryoprotected in 20% sucrose in 0.1 M PBS at 4°C for ~3 days. Brains were sectioned coronally (30 µm) with a cryostat. Sections were blocked in 0.1M PBS with 5% donkey serum and 0.2% Triton-X at room temperature and incubated in primary antibodies overnight in 5% normal donkey serum

with 0.2% Triton-X. The following primary antibodies used were at a 1:1000 dilution: rabbit anti-CTR (ab11042, Abcam, Cambridge, MA, USA), mouse anti-NeuN (MAB377, Millipore, Billerica, MA, USA), chicken anti-glial fibrillary acidic protein (GFAP, AB5541, Millipore, Billerica, MA, USA), and goat anti-choline acetyltransferase (ChAT, AB144P, Millipore, Billerica, MA, USA) (Wang and Morales, 2009; Lopez et al., 2012; Mietlicki-Baase et al., 2015b; Saunders et al., 2015). Following a PBS rinse, sections were incubated in appropriate donkey Alexa Fluor 488, 594, and AMCA secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) at room temperature (all secondary antibodies 1:500 in 5% normal donkey serum with 0.2% Triton-X). Sections were mounted on slides and visualized with a Leica SP5 X confocal microscope using objectives and the 405, 488, and 594 laser lines. All images were collected sequentially to avoid contamination of signals from other fluorophores.

Colchicine treatment and co-localization of CTR and GABAergic markers

To enhance labeling for glutamate decarboxylase 67 (Gad67), a GABAergic marker, a rat (n=1) was injected with colchicine (400µg, dissolved in 4µl of DMSO), a neurotoxin that blocks neurotransmission, directly in the lateral ventricle (Halasy et al., 2004; Wang et al., 2014). Twenty-eight hours after colchicine treatment, the rat was anesthetized with the surgical dose of KAX used in the lateral ventricle cannulation, perfused transcardially with ice-cooled 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borate buffer of pH 9.5. The brain was removed and immersed in 4% paraformaldehyde containing 12% sucrose for 20–24h at 4^{o}C. The brain was then blocked transversely at the level of the caudal midbrain, and the block was flash-frozen in dry-ice cooled hexane before being sectioned frozen on a sliding microtome (transverse plane, 30 µm thickness, 5 series). Sections were stored in antifreeze solution

(30% ethylene glycol, 20% glycerol in 0.02 M potassium phosphate-buffered saline—KPBS) at -20℃ until further processing. Sections were incubated in primary antibodies at 4°C overnight in KPBS containing 1% donkey serum and 0.1% Triton X-100. The following primary antibodies were used: rabbit anti-CTR (1:1000, ab11042, Abcam, Cambridge, MA, USA) and mouse anti-GAD67 (1:10000, MAB5406, Millipore, Billerica, MA, USA) (Deidda et al., 2015; Mietlicki-Baase et al., 2015b). Following washes in KPBS, sections were incubated in appropriate secondary antibodies at 4°C overnight diluted at 1:500 in KPBS containing 0.1% Triton X-100 (donkey AffiniPure Cy3 and Alexa Fluor 488, Jackson Immunoresearch, West Grove, PA, USA). Following washes in KPBS, sections were mounted onto glass slides, allowed to dry, and coverslipped, using 50% glycerol in KPBS mountant. Coverslip edges were sealed with clear nail polish. Photomicrographs were acquired as optical slices using a Zeiss LSM 700 UGRB Confocal System (controlled by Zeiss Zen software). All photomicrographs in the figures are oriented such that: up = dorsal, down = ventral, left = medial, right = lateral.

Behavioral testing

General procedures

Drug injections were made just prior to the onset of the dark cycle unless otherwise specified. For experiments measuring *ad libitum* food intake, weights of food hoppers were recorded to the nearest 0.1g and food spillage is accounted for in cumulative food intake measurements. Food intake was recorded at 1, 3, 6, and 24h after injection, while body weight was measured at 0 and 24h after injection, except where noted. Injections were administered using a within-subjects counterbalanced design and were separated by at least 72h.

Intra-LDTg amylin and salmon calcitonin dose-response feeding experiments

To assess the dose-response of amylin receptor activation in the LDTg on food intake and body weight gain, rats (n=10) received unilateral LDTg injections of amylin (0, 0.2, 0.4, and 0.8μg; 100 nl aCSF) shortly before the onset of the dark cycle. The doses of amylin for this experiment were selected based on previous dose response experiments in the 3rd ventricle and VTA (Rushing et al., 2000; Mietlicki-Baase et al., 2015a). In order to compare the hypophagic effects of LDTg administration of amylin with the long acting amylin receptor agonist sCT, we injected sCT unilaterally into the LDTg just before the onset of the dark cycle in a separate cohort of rats (n=6). The sCT doses for this series of experiments (0, 0.01, 0.04, and 0.1μg; 100 nl aCSF) were selected based on previous dose response experiments in the 3rd ventricle and VTA (Mietlicki-Baase et al., 2013b). Given that previous studies have shown that lower doses of sCT are required to produce a feeding effect compared to amylin (Lutz et al., 2000; Mietlicki-Baase et al., 2013b; Mietlicki-Baase et al., 2015a; Mietlicki-Baase et al., 2015b), the doses of amylin were considerably lower than that of sCT.

For meal pattern experiments, rats (n=5) were housed in a custom-made automated feedometer system that consists of hanging wire mesh cages with a small hole allowing access to a food cup that rests on an electronic scale (Hayes et al., 2011b; Kanoski et al., 2012b; Mietlicki-Baase et al., 2013b). Computer software (LabView) recorded the weights of the food cups every 10s for 24h. A meal was defined as at least 0.25g of food intake with at least 10 minutes in between feeding bouts (Hayes et al., 2011b; Kanoski et al., 2012b; Mietlicki-Baase et al., 2013b). Meal patterns were assessed at 24h post-LDTg injection (0, 0.01, 0.04, or 0.1μg sCT).

To test if nausea/malaise contributes to the intake suppression following LDTg amylin receptor activation by measuring pica, rats (n=6) were given access to both chow and kaolin clay (Research Diets, New Brunswick, NJ, USA) for ~1 week prior to the experiment. Chow intake, kaolin clay intake, and body weight were measured at 24h after injection of sCT (0, 0.01, 0.04, or 0.1µg) into the LDTg.

Intra-LDTg amylin receptor activation sucrose self-administration experiment

Experiments were conducted in ventilated, sound-attenuating operant chambers (Med Associates, St Albans, VT, USA). Rats (n=8) were initially food restricted to ~80% of daily intake and trained to lever press for 45 mg sucrose pellets (Research Diets) on a fixed ratio 1 (FR1) schedule of reinforcement during 1 h operant sessions. Once animals achieved stable responding for sucrose (defined as <20% variation in responding over three consecutive days) on the FR1 schedule of reinforcement, the response requirement was increased to FR3 for two days, followed by three days on FR5. Animals were then given access to *ad libitum* chow and maintained on FR5 for an additional seven days. Motivation to self-administer sucrose pellets was then assessed using a progressive ratio (PR) schedule of reinforcement. Under a PR schedule, the response requirement for the *i*th reinforcement of each subsequent delivery of a sucrose pellet increases exponentially, according to the formula $R(i) = [5e^{0.2i} - 5]$, until the rat fails to meet the requirement in a 30 minute time period (Hopkins et al., 2012). Using a within-subjects design, rats were injected with aCSF (100 nl), amylin (0.4µg; 100 nl), or sCT (0.04µg; 100 nl) into the LDTg 30 minutes prior to a PR self-administration test session. We chose these doses based on previous literature showing that lower doses of sCT are needed to produce a feeding effect compared to amylin (Lutz et al., 2000; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2015a; Mietlicki-Baase et al., 2015b).

Treatments were counterbalanced and test days were separated by at least 2 days of responding for sucrose on the FR5 schedule of reinforcement. The responding of one rat declined prior to completion of the experiment due to equipment malfunction, and he was subsequently removed from behavioral analyses.

Intra-LDTg acute amylin receptor blockade feeding experiment

We examined whether acute pharmacological blockade of LDTg amylin receptors was sufficient to attenuate the intake suppressive effects of a systemic sCT injection. The amylin receptor antagonist, AC187, was bilaterally injected in the LDTg (0.8µg/100nl/hemisphere; dose chosen from pilot experiments to be subthreshold for food intake effect on its own) 1h prior to the onset of the dark cycle (Mietlicki-Baase et al., 2013b). Fifteen minutes prior to the onset of the dark cycle, rats (n=11) were injected systemically with the amylin receptor agonist, sCT (5 µg/kg, IP), and subsequent food intake and body weight were measured.

Intra-LDTg amylin receptor knockdown experiment

A validated AAV1-shRNA construct for CTRa/b knockdown was used as previously described (Mietlicki-Baase et al., 2015b). Rats were maintained on chow for 1 week prior to surgery to establish baseline intakes and body weight and were divided into two weight-matched groups. Animals were assigned to receive a bilateral LDTg injection (200 nl/hemisphere) of either an AAV control vector expressing GFP (AAV-Control, n=7) or the AAV-shRNA for the CTR-a/b expressing GFP (AAV-CTR KD, n=7). Beginning post-surgery day 1, food intake, food spillage and body weight were measured every 48h for a total of 30 days (days 1-31 post-viral injection). One rat was removed from the behavioral analyses due to technical errors in data collection. After conclusion of the behavioral study, rats were anesthetized with a surgical dose of KAX

used in the LDTg cannulation (IM) and euthanized by decapitation. The brains were rapidly removed and flash frozen. Correct placement of AAV injections was verified by collecting a section containing the LDTg (30 μm) from each brain and visualizing GFP. Micropunches of the LDTg were collected and the LDTg CTRa knockdown was confirmed with qPCR, with GapDH as the internal control.

Intra LDTg GABA receptor blockade feeding experiment

To test the role of GABA receptors in mediating LDTg amylin-mediated intake suppression, a cocktail composed of bicuculline (GABA-A receptor antagonist, 100 ng; 100 nl) and saclofen (GABA-B receptor antagonist, 500 ng; 100 nl) was unilaterally injected in the LDTg (100 nl, 50% DMSO in aCSF) 30 minutes prior to the onset of the dark cycle. Fifteen minutes prior to the onset of the dark cycle, rats (n=8) were unilaterally injected with sCT (0.04μg; 100 nl, aCSF vehicle) in the ipsilateral LDTg, and food intake and body weight were measured.

Statistical Analyses

All data are represented as mean ± SEM. The α level was set to $p \leq 0.050$ for all studies. Statistical analyses were performed using Statistica 13.0 (StatSoft). For all feeding and body weight studies, binned data were analyzed using separate repeated measures ANOVAs that accounted for the within-subjects experimental design, while assessing between-subjects effects (drug treatment, AAV condition) when applicable, except when noted. Body weight for the GABA-A/B receptor blockade experiment was analyzed as a repeated measures one-way ANOVA. Sucrose self-administration was analyzed using separate ANOVAs for each behavioral measure, accounting for the within-subject experimental design. CTR/RAMP expression was analyzed, relative to CTRb and RAMP3, using separate ANOVAs for each gene that accounted for the within-

subject experimental design. For the CTR knockdown experiment, gene expression was analyzed using an ANOVA that accounted for the between-subject experimental design. Statistically significant effects were probed using Student-Neuman-Keuls *post hoc* analyses or planned comparisons when noted.

Results

The components of the amylin receptor complex are expressed in the LDTg

Amylin receptors are formed by heteromeric interaction between one of two G_s/G_q-coupled calcitonin receptors (CTRa or CTRb) and one of three receptor activity-modifying proteins (RAMP1-3) (Poyner et al., 2002; Morfis et al., 2008). Although the LDTg binds amylin (Sexton et al., 1994), no studies to date have examined expression of the amylin receptor complex within this nucleus. Therefore, we used quantitative real-time PCR to determine expression of the components of the amylin receptor (CTRa/b, RAMP1-3) in the LDTg, and found that both CTRs and all three RAMPs are indeed expressed in this nucleus (n=6). CTRa gene expression is approximately 5-fold greater than expression of CTRb, although this does not reach statistical significance ($F_{1,3}$=4.04, p=0.14; Figure 1A). Gene expression of RAMP1 is approximately 2-fold greater than the expression of RAMP2 ($F_{2,6}$=13.04, p<0.01; posthoc test, p<0.05) and approximately 13-fold greater than the expression of RAMP3 (posthoc test, p<0.01; Figure 1B). These findings are consistent with data from the AP and VTA, which also show higher expression of CTRa compared to CTRb and abundant RAMP1 expression (Lutz, 2012; Mietlicki-Baase et al., 2013b).

Next, we performed immunohistochemical (IHC) analyses to label cells that express CTR (n=6). Our data show labeling throughout the rostral-caudal axis of the LDTg, with particularly dense labeling in the caudal LDTg (8.6-9.1mm posterior to bregma), providing evidence of amylin receptor expression at the protein level. Representative images from the caudal LDTg are shown in Figure 1C-D. Together, data in Figure 1 show that components of the amylin receptor complex are expressed in the

LDTg at the gene and protein levels. Due to the dense CTR expression observed in the caudal LDTg, we targeted this subregion in our behavioral experiments.

LDTg amylin suppresses cumulative chow intake and body weight

As all components of the amylin receptor are expressed in the LDTg, this nucleus may mediate, in part, the negative energy balance effects of amylin. To test whether activation of amylin receptors in the LDTg by the native amylin peptide is sufficient to decrease food intake, rats (n=10) were unilaterally injected in the LDTg with amylin (0, 0.2, 0.4, and 0.8μg; 100 nl aCSF; see Figure 2A for representative injection placement) and subsequent chow intake and body weight change were recorded over a 24h period. Injection of amylin in the LDTg dose-dependently decreases food intake over 6h ($F_{3,27}$≥3.00, $p<0.05$; Figure 2B) but not 24h food intake or body weight change ($F_{3,27}$≤2.32, $p>0.05$; Figure 2C). Consistent with previous reports of amylin-induced hypophagia at early time points (Lutz et al., 1998a; Mietlicki-Baase et al., 2015a), all doses of amylin administered to the LDTg suppress chow intake at 1h ($p<0.01$), but only the highest dose of amylin (0.8μg) suppresses chow intake at 3 and 6h after injection ($p<0.05$) compared to aCSF treatment.

LDTg amylin receptor activation suppresses cumulative chow intake and body weight

To determine whether pharmacological LDTg amylin receptor activation with the long-acting amylin receptor agonist sCT produces more durable and more potent hypophagic effects, sCT (0, 0.01, 0.04, or 0.1μg), was injected unilaterally into the LDTg and subsequent chow intake and body weight change were recorded over a 24h period (n=6). Notably, the two lower doses of sCT, 0.01 and 0.04 μg, are subthreshold for prolonged effects on food intake and body weight when applied to the 3rd ventricle (Mietlicki-Baase et al., 2013b). Results of this study show that intra-LDTg amylin

receptor activation with sCT dose-dependently suppresses chow intake at 1, 3, 6, and 24h after injection ($F_{3,12}$≥3.66, p<0.05; Figure 2B). Post-hoc analyses reveal that all 3 doses of sCT produce a significant suppression of chow intake, compared to aCSF vehicle treatment, at 1h (p<0.01) and 6h (p<0.05) after injection. Additionally, the two highest sCT doses (0.04 and 0.1 µg) decrease food intake at 24h after injection (p<0.05). Body weight gain over the 24h post-injection is also significantly reduced by intra-LDTg administration of 0.04 or 0.1 µg sCT ($F_{3,12}$=11.00, p<0.01; compared to aCSF, p<0.05; Figure 2C). These data indicate that LDTg amylin receptor activation dose-dependently suppresses chow intake and body weight over 24h. Importantly, behavioral data from animals (n=3) with missed LDTg cannula placements does not show hypophagia after amylin receptor activation (See Figure A3 in Appendix; ($F_{1,3}$<0.6, p>0.64). Taken together and consistent with previous literature (Lutz et al., 2000; Reidelberger et al., 2002), LDTg amylin receptor activation with sCT results in more potent and longer-lasting hypophagic effects than LDTg administration of native peptide amylin.

LDTg amylin receptor activation suppresses meal size

To evaluate the behavioral mechanisms driving the hypophagia following LDTg amylin receptor activation, meal patterns were analyzed (n=5). Unilateral injection of sCT in the LDTg at doses effective for reducing overall intake (0, 0.01, 0.04, or 0.1µg) significantly suppresses meal size 24h after injection ($F_{3,15}$=5.18, p<0.01; Figure 3A). Post-hoc analyses reveal that administration of the two highest doses, 0.04 and 0.1 µg sCT, significantly decreases meal size compared to aCSF treatment (p<0.05), consistent with the established role of amylin as a satiation signal (Lutz, 2012; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2015b). Along with this suppression in meal size, intra-

LDTg administration of sCT also reduces meal duration at all doses tested ($F_{3,15}$=5.51, p<0.01; p<0.05, compared to aCSF treatment; Figure 3B). LDTg amylin receptor activation increases latency to first meal ($F_{3,15}$=4.90, p<0.05; Figure 3C) at the highest dose (p<0.05, compared to aCSF treatment), which indicates a decreased motivation to initiate feeding. Intra-LDTg administration of sCT decreases meal number over 24h after injection ($F_{3,15}$≥3.77, p<0.05: Figure 3D), but only with the highest dose, 0.1 µg sCT (p<0.05, compared to aCSF treatment). These data show that LDTg amylin receptor activation reduces food intake predominately via suppression of meal size rather than meal number. Importantly, this reduction in meal size is concomitant with a decrease in meal duration, which may be a consequence of reduced within-meal motivation to continue to feed and/or reflect the normal physiological characteristics of amylin's effects on the behavioral satiation sequence.

LDTg amylin receptor activation attenuates motivation for a palatable food

Given that the LDTg is a reward-relevant nucleus (Schmidt et al., 2009; Dickson et al., 2011), we next tested the hypothesis that LDTg amylin receptor activation attenuates motivated feeding as measured by sucrose self-administration on a progressive ratio (PR) schedule of reinforcement (n=8). Unilateral injection of either the native peptide amylin (0.4µg) or amylin receptor agonist sCT (0.04 µg) into the LDTg significantly suppresses active lever responses for sucrose ($F_{2,14}$=11.52, p<0.01: Figure 4A), breakpoint ($F_{2,14}$=11.26, p<0.01: Figure 4B), and sucrose pellets earned ($F_{2,14}$=7.72, p<0.01; Figure 4C) compared to aCSF treatment. Notably, there is no difference between treatments on inactive lever responding ($F_{2,14}$=1.13, p=0.35; Figure 4A). These data indicate that LDTg amylin receptor activation, both with the potent amylin receptor

agonist, sCT, and the native ligand, amylin, reduces motivation to self-administer a palatable food (i.e. sucrose).

LDTg amylin receptor activation does not produce malaise

To determine if nausea/malaise contributes to the intake suppression following central amylin receptor activation, pica was measured after intra-LDTg sCT administration. Pica is the ingestion of non-nutritive substances, such as kaolin clay, and is a well-established model for nausea/malaise in non-vomiting species such as the rat (Mitchell et al., 1977; Takeda et al., 1993; Yamamoto et al., 2002; Andrews and Horn, 2006; Kanoski et al., 2012a). The same doses of sCT used in the previous behavioral studies (0, 0.01, 0.04, or 0.1μg) were injected unilaterally in the LDTg, and intakes of chow and kaolin clay were measured 24h after injection (n=6). Intra-LDTg sCT does not increase kaolin intake at any dose ($F_{3,15}$=0.98, p=0.45; Figure 4D), but all three doses significantly suppress chow intake at 24h compared to aCSF treatment ($F_{3,15}$=7.93, p<0.01; post-hoc test, p<0.05, Figure 4E). These data suggest that the hypophagia and decreased motivation to feed following intra-LDTg amylin receptor activation are likely not due to induction of nausea/malaise.

LDTg amylin receptor blockade attenuates the intake suppressive effects of peripheral amylin receptor activation

Given that pharmacological activation of amylin receptors directly in the LDTg suppresses food intake, the ability of peripherally administered amylin or amylin receptor agonists to access the CNS, and act specifically within the LDTg, is a key consideration in the development of amylin-based anti-obesity pharmaceuticals and denotes potential pre-clinical relevance in animal models and clinical relevance in humans. Thus, to begin

to address this critical question using a pre-clinical rodent model, we evaluated whether the intake- and body weight-suppressive effects of systemic sCT (5 μg/kg, IP) would be attenuated by acute LDTg amylin receptor blockade. We intentionally chose a dose of the amylin receptor antagonist AC187 that is subthreshold for an effect on feeding when delivered bilaterally within the LDTg (0.8 μg/hemisphere; n=11) so as not to have competing orexigenic and anorexic behavioral responses. As expected, systemic administration of sCT significantly suppresses cumulative chow intake at 3, 6, and 24h after injection (main effects of sCT, $F_{1,10} \geq 11.31$, $p<0.01$; planned comparisons of aCSF/sCT versus aCSF/saline or AC187/saline at 3, 6, and 24h, $p<0.05$; Figure 5A). A significant interaction between sCT and AC187 occurs at 6h and 24h after injection ($F_{1,10} \geq 5.20$, $p<0.05$); post hoc analyses reveal that pre-treatment with intra-LDTg AC187 significantly attenuates the intake-suppressive effects of peripheral sCT at 24h ($p<0.05$). Systemic administration of sCT also decreases 24h body weight gain ($F_{1,10}=20.30$, $p<0.01$, main effect of sCT; Figure 5B). Treatment with aCSF/sCT suppresses 24h body weight gain compared to aCSF/saline and AC187/saline conditions (planned comparisons, $p<0.05$). Importantly, amylin receptor blockade alone (AC187/saline) does not significantly increase chow intake at any time point (no main effects of AC187, $F_{1,10}<1.61$, $p>0.2$) or body weight (no main effect of AC187, $F_{1,10}=1.56$, $p>0.2$). These data show that intra-LDTg amylin receptor blockade attenuates the intake-suppressive effects of a systemically delivered amylin receptor agonist, suggesting the potential pre-clinical relevance of LDTg amylin receptor signaling.

Knockdown of calcitonin receptors in the LDTg increases chow intake and body weight

In order to determine if endogenous LDTg amylin receptor signaling is physiologically required for the normal day-to-day control of energy balance, an adeno-

associated virus of serotype 1 (AAV1) that encodes a short hairpin RNA (shRNA) to knockdown CTR, the core component of the amylin receptor (AAV-CTR KD), or an empty vector control (AAV-Control) (Mietlicki-Baase et al., 2015b) was injected bilaterally into the LDTg (200nl/hemisphere). Compared to AAV-Control animals, AAV-CTR KD decreases LDTg CTRa expression by approximately 67% (Figure 6A, $F_{1,10}$=5.43, $p<0.05$). Representative GFP visualization of viral targeting and spread from a separate cohort of animals sacrificed two weeks after bilateral LDTg viral injection (n=3/viral condition) is shown in Figure 6B.

Animals with LDTg amylin receptor knockdown show a sustained elevation in body weight compared to the AAV-Control rats (Figure 6C). ANOVAs show that AAV-CTR KD animals weigh more than AAV-Control animals, either approaching ($F_{1,12}\geq3.30$, $p<0.1$) or reaching ($F_{1,12}\geq4.75$, $p<0.05$) statistical significance on any given experimental test day, beginning 3 days after viral injection. When analyzed as cumulative body weight gain from D0 to D31, AAV-CTR KD produces a significant increase in body weight gain compared to AAV-Control ($F_{1,12}$=4.73, p=0.050; Figure 6E).

AAV-CTR KD treatment causes small increases in 48h binned food intake (Figure 6D). ANOVAs show that AAV-CTR KD animals eat significantly more in 48h bins than AAV-Control animals on days 7-9 and 29-31 ($F_{1,12}\geq5.78$, $p<0.05$), with a trend for significance ($F_{1,12}\geq3.50$, $p<0.1$) on days 1-3, 9-11, and 19-21. When graphed cumulatively from day 0 to day 31, AAV-CTR KD rats have a trend for increased cumulative intake compared to AAV-Control rats ($F_{1,12}$=3.92, $p<0.1$; Figure 6F). Together, these data show that endogenous amylin accesses the LDTg and establish a

physiological role for LDTg amylin receptor signaling in the normal control of food intake and body weight regulation.

LDTg amylin receptors are expressed on GABAergic neurons

Next, we performed immunohistochemistry to determine the phenotype of amylin receptor-expressing cells in the LDTg. Sections were labeled for the amylin receptor (CTR), NeuN (a neuronal marker), and GFAP (a glia/astrocyte marker). CTR expressing cells in the LDTg co-localize exclusively with NeuN (Figure 7A), suggesting that amylin receptor-expressing cells in the LDTg are primarily, if not exclusively, neuronal (n=3).

To begin to evaluate the phenotype of LDTg CTR-expressing neurons, further IHC experiments tested if the CTR-positive neurons within the LDTg are cholinergic or GABAergic, as these represent classic LDTg neurotransmitter phenotypes (Wang and Morales, 2009). Results indicate that CTR in the LDTg does not co-localize with ChAT, a marker for cholinergic neurons (Figure 7B; n=6). After colchicine treatment (Wang et al., 2014), 13.7% of CTR neurons in the LDTg, specifically in the caudal LDTg (-8.6mm to -9.1mm from bregma) co-localize with the GABAergic neuronal marker, Gad67 (Figure 7C; n=1). These data suggest that at least a portion of amylin receptor-expressing cells in the LDTg are GABAergic neurons, though we cannot rule out the possibility that colchicine treatment did not result in labeling of all Gad67 cells.

Intra-LDTg GABA-A/B receptor blockade reverses the intake-suppressive effects of LDTg amylin receptor activation

As our data show that LDTg amylin receptor signaling regulates food intake and body weight, and that a portion of LDTg amylin receptor-expressing cells are GABAergic, we next evaluated the hypothesis that LDTg GABA receptor signaling is

downstream of LDTg amylin receptor activation and mediates LDTg amylin-induced hypophagia. To test this hypothesis, a cocktail composed of the GABA-A receptor antagonist bicuculline (100 ng) and the GABA-B receptor antagonist saclofen (500 ng) was unilaterally injected in the LDTg at doses subthrehold for an effect on feeding (100 nl, 50% DMSO in aCSF) followed by a unilateral injection of sCT (0.04µg; 100 nl, aCSF vehicle) in the ipsilateral LDTg; subsequent chow intake and body weight change were measured (n=8).

For cumulative chow intake (Figure 8A), repeated measures ANOVAs show a significant main effect of sCT at all timepoints ($F_{1,7} \geq 8.30$, $p<0.05$) and a significant main interaction between sCT and GABA receptor blockade at 24h after injection ($F_{1,7}=7.47$, $p<0.05$). Specifically, chow intake following 50% DMSO/sCT is significantly suppressed at 1 and 24h after injection, compared to all other conditions (planned comparisons, $p<0.05$). Importantly, intra-LDTg GABA-A/B receptor blockade does not affect feeding on its own ($F_{1,7}<4.70$, $p>0.1$), but reverses the intake-suppressive effects of intra-LDTg sCT at 24h after injection (GABA-A/B receptor antagonists/sCT versus 50% DMSO/sCT, $p<0.05$; versus 50% DMSO/aCSF, $p>0.4$). Based on the feeding data, we analyzed the body weight change (Figure 8B) as a one-way repeated measures ANOVA by treatment ($F_{3,21}=7.77$, $p<0.01$). Body weight gain following 50% DMSO/sCT treatment is significantly suppressed compared to all other treatments ($p<0.01$). Importantly, GABA receptor blockade alone (GABA-A/B receptor antagonists/aCSF) does not significantly alter body weight ($p>0.9$) compared to 50% DMSO/aCSF treatment. These data show that intra-LDTg GABA-A/B receptor blockade attenuates the anorexia produced by an intra-LDTg amylin receptor agonist.

Discussion

The current obesity epidemic (Ogden et al., 2012) highlights the urgent need to understand the neuroendocrine signals and neurobiological substrates that regulate energy balance, which in turn will inform the identification of novel opportunities for obesity pharmacotherapies. Recent attention has focused on targeting the amylin system for treating obesity, as the amylin analogue pramlintide is FDA-approved for diabetes and also decreases food intake and body weight in obese patients (Edelman et al., 2008; Halford et al., 2010). Although research on amylin's effects on energy balance has predominately focused on hindbrain and hypothalamic structures [see (Rushing, 2003; Lutz, 2012; Mietlicki-Baase and Hayes, 2014) for review], *in vitro* radiography data show amylin binding sites are found throughout the brain (Sexton et al., 1994), suggesting the likelihood of more distributed effects. The LDTg of the caudal midbrain represents one such amylin binding site; this nucleus receives information from and projects to several hindbrain, midbrain, and forebrain structures important for food intake, body weight regulation, and reward (Cornwall et al., 1990). Our experiments here show that the components of the amylin receptor complex are expressed in the LDTg and that amylin receptor signaling in the LDTg is important for the control of food intake and body weight regulation. Additionally, our data identify a portion of LDTg amylin receptor-expressing cells as GABAergic neurons that we speculate may be interneurons. These findings highlight the LDTg as a potential energy balance hub and show that this nucleus is of potential pre-clinical relevance as a neural substrate that can be targeted for future amylin-based pharmacotherapies for obesity.

Despite the fact that the LDTg receives information from and projects to a number of feeding- and reward-relevant nuclei throughout the brain (Cornwall et al.,

1990) and expresses receptors for a variety of feeding peptides (e.g. amylin, ghrelin, glucagon-like peptide-1, peptide YY) (Merchenthaler et al., 1999; Parker and Herzog, 1999; Dickson et al., 2011; Cabral et al., 2013), little attention has been paid to this nucleus for its role in energy balance control. The current data showing that LDTg amylin receptor activation suppresses food intake and body weight is highly novel and consistent with the satiating properties following systemic or intracerebroventricular administration of amylin [see (Lutz, 2012; Hayes et al., 2014a) for review]. Importantly, the suppression in food intake by LDTg amylin receptor activation is not likely due to nausea/malaise as LDTg amylin receptor activation does not produce pica, suggesting the specificity of the energy balance effects. Two additional explanations underlying the body weight changes following LDTg amylin receptor activation are decreases in intestinal food weight and/or reductions in prandial drinking. Future studies should examine whether LDTg amylin signaling directly impacts fluid intake in the absence of food, as well as any potential effects on intestinal food weight. The aforementioned experiments utilize the amylin receptor agonist sCT, which binds irreversibly with high affinity to amylin receptors but also with low affinity to calcitonin receptors (Christopoulos et al., 1999; Lutz et al., 2000; Tilakaratne et al., 2000). In contrast, amylin itself binds with moderate affinity to amylin receptors and with very low affinity to calcitonin receptors (Christopoulos et al., 1999; Morfis et al., 2008). Furthermore, while we show evidence of gene expression of the amylin receptor complex in the LDTg, it is important to point out that the qPCR micropunch data cannot establish whether both components of the amylin receptor are expressed in the same cell. However, given that intra-LDTg amylin administration suppresses food intake and body weight in a dose-dependent manner, the collective body of data presented here suggest that complete amylin

receptors are likely expressed in the LDTg and amylin receptor signaling is likely mediating the observed hypophagic response.

The within-meal intake inhibitory effects of LDTg amylin signaling may be explained by a reduction in the rewarding value of the ongoing meal. Indeed, we show that LDTg amylin receptor activation not only suppresses the size of the meal, but also produces a concomitant decrease in meal duration, as well as a decrease in motivation to work for a palatable sucrose reward. The LDTg is reciprocally connected to both the NTS and VTA (Cornwall et al., 1990). Given the role of the NTS in meal size control [see (Moran, 2009; Grill and Hayes, 2012) for review] and the VTA in reward processing [see (Narayanan et al., 2010; DiLeone et al., 2012) for review], the suppression of meal size observed after LDTg amylin receptor activation likely involves amylinergic modulation of NTS-LDTg-VTA neural processing. However, future systematic neuroanatomical studies are needed to confirm that amylin receptor-expressing LDTg neurons impinge on this proposed NTS-LDTg-VTA circuitry through putative LDTg GABAergic inhibition of the NTS-LDTg-VTA polysynaptic communication. Alternatively, the decreased PR responding may be a secondary response to LDTg amylin receptor signaling inducing satiation signaling more generally and potentially independent of reward signaling.

Previous studies have established a role for the LDTg in reward processing for drugs of abuse and natural rewards (e.g. food, sex) through modulation of VTA dopaminergic cell firing (Floody and Cramer, 1986; Lodge and Grace, 2006; Schmidt et al., 2009; Dickson et al., 2011; Steidl et al., 2015). Our findings extend this literature on the role of the LDTg in modulating feeding behavior and energy balance, and provide novel evidence to suggest that LDTg signaling modulates the rewarding value of the

ongoing meal. Given that activation of a LDTg-VTA pathway and its downstream targets can promote feeding and reward-associated behaviors, such as conditioned place preference and cocaine seeking (Schmidt et al., 2009; Dickson et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012; Lammel et al., 2012), amylin receptor activation of inhibitory GABA neurons in the LDTg may decrease VTA dopaminergic cell firing, ultimately leading to hypophagia and a reduction in motivated feeding. Our IHC and behavioral data provide converging evidence in support of this hypothesis. We speculate that LDTg amylin receptors may be expressed on putative GABAergic interneurons, suggesting that LDTg amylin receptor activation could result in local inhibition of a variety of output neural pathways, including those projecting to the VTA. Future studies should therefore examine whether LDTg amylin receptor activation suppresses VTA dopaminergic cell activity in response to a food reward, and whether this outcome is in fact LDTg-GABA mediated.

Arguably one of the most important findings from the current data set is that acute blockade of LDTg amylin receptors attenuates the intake-suppressive effects of a systemic amylin receptor agonist. Though a significant attenuation of the intake-suppressive effects is not observed until the 24h timepoint, these data are comparable to a similar experiment performed in the VTA (Mietlicki-Baase et al., 2013b) in which effects were also only observed at 24h. In contrast to the VTA and LDTg, previous reports have shown that systemically delivered amylin agonists are able to activate AP amylin receptors more rapidly (Roth et al., 2009; Mietlicki-Baase and Hayes, 2014). Thus, there appears to be a temporal difference in systemic amylin agonists' action in distributed nuclei throughout the neuraxis that requires further investigation. Nevertheless, the data suggest that amylin receptor agonists administered systemically

can access the LDTg, and thus LDTg amylin receptors may represent a pre-clinically relevant CNS population that can be targeted by peripherally-administered amylin receptor ligands for the treatment of obesity. Importantly, the dose of AC187 utilized here was selected to be subthreshold for an effect on food intake when administered in the LDTg. However, future experiments should utilize different doses of AC187 on its own in the LDTg to examine the effect of endogenous amylin on food intake, body weight, and motivated behavior in acute studies. Furthermore, the longer-term physiological role of LDTg amylin receptor signaling for energy balance control is supported by our study examining the effects of LDTg amylin receptor knockdown. Virogenetic knockdown of LDTg CTR increased body weight and food intake, suggesting that endogenous amylin can access the LDTg and that LDTg amylin receptors exert chronic control over energy balance. Interestingly, binned increases in food intake were modest compared to binned increases in body weight, suggesting an unexplored contribution of decreased energy expenditure following LDTg amylin receptor knockdown. Therefore, future experiments should examine how amylin signaling in the LDTg affects energy expenditure.

The novel findings here support the hypothesis that amylin receptor signaling in the LDTg is important for food intake and body weight regulation. These data highlight the importance of focusing further attention on this understudied nucleus in the field of obesity research. We have identified a subset of amylin receptor expressing cells in the LDTg are GABAergic neurons, which allows for future dissection of the downstream neurons and nuclei that are presumably inhibited by LDTg amylin receptor activation. As the LDTg also expresses receptors for other energy balance-relevant hormones (Merchenthaler et al., 1999; Parker and Herzog, 1999; Dickson et al., 2011; Cabral et al.,

2013), future studies should explore how amylin signaling in the LDTg potentially interacts with other feeding-related signals to exert integrated control of energy balance and food reward.

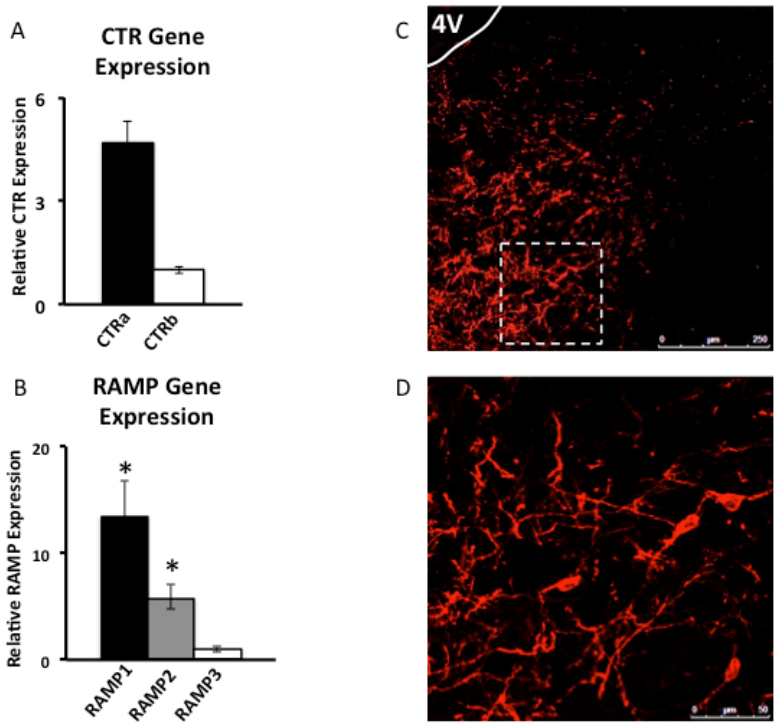

Figure 1. The components of the amylin receptor complex are expressed in the LDTg. Micropunches of LDTg-enriched tissue (n=6) show expression that gene expression of CTRa is ~5 fold higher than CTRb (A), and gene expression of RAMP1 is ~2-fold higher than RAMP2 and ~13-fold higher than RAMP3 (B). Immunohistochemical data using CTR to label amylin receptor-expressing cells (n=6) show dense labeling of cell bodies and projections in the caudal LDTg (C, D). The dotted box in C (20x) represents the field of view in D (20x with a 2x optical zoom). * indicates significance by repeated measures ANOVA ($p<0.05$).

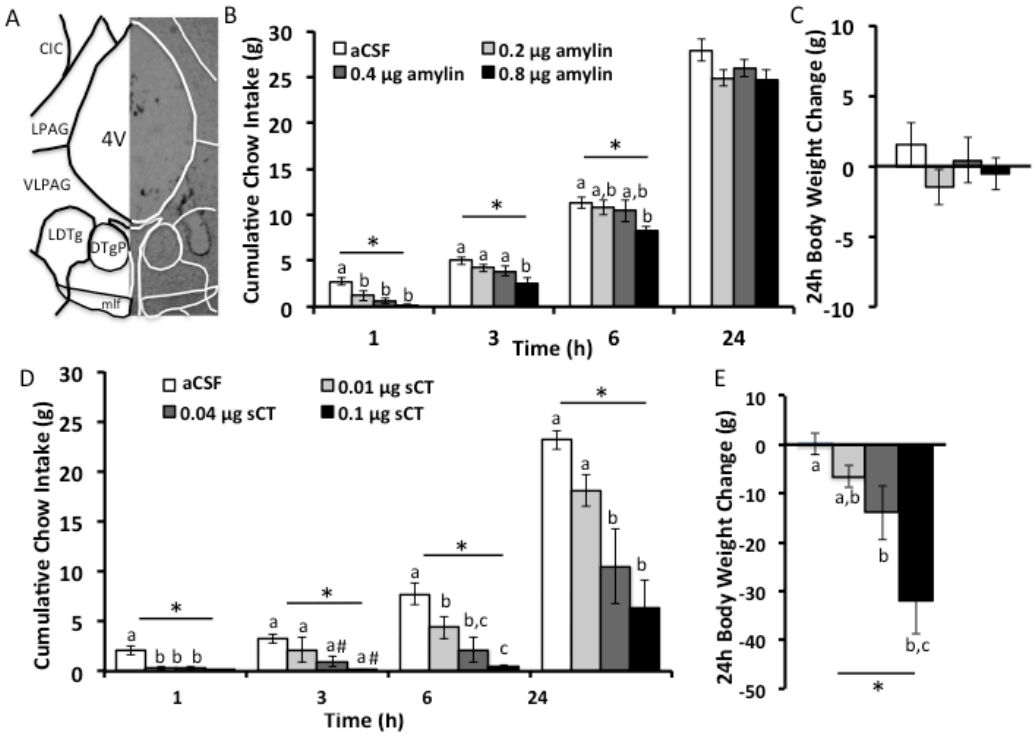

Figure 2. Intra-LDTg amylin receptor activation dose dependently suppresses chow intake and body weight. Amylin was unilaterally injected into the LDTg in a counterbalanced within-subjects design at the onset of the dark cycle using the following doses: 0 (aCSF), 0.2, 0.4, and 0.8 µg (n=10). A representative image of the LDTg injection site from a 35 µm thick section is shown (A). These doses of amylin dose-dependently decrease chow intake over 6h but have no effect on 24h chow intake (B) or body weight change (C). The key in B also applies to C. In a separate cohort of rats, the amylin receptor agonist sCT was unilaterally injected into the LDTg in a counterbalanced within subjects design at the onset of the dark cycle using the following doses: 0 (aCSF), 0.01, 0.04, and 0.1 µg (n=6). These doses of sCT suppress chow intake at every time point tested over 24h (D) and decrease 24h body weight gain (E). * indicates significance by repeated measures ANOVA ($p<0.05$), # indicates a trend for significance by post-hoc Neuman-Keuls ($p<0.1$). Different letters are significantly different from each other ($p<0.05$) according to post-hoc tests. The key in D also applies to E. Atlas image is -8.7 mm from bregma, based on Paxinos & Watson, 2007. 4V = 4th ventricle, CIC = central nucleus inferior colliculus, DTgP = Dorsal tegmental nucleus pericent, LDTg= lateral dorsal tegmental nucleus, LPAG = lateral periaqueductal gray, mlf = medial longitudinal fasciculus, VLPAG = ventral lateral periaqueductal gray.

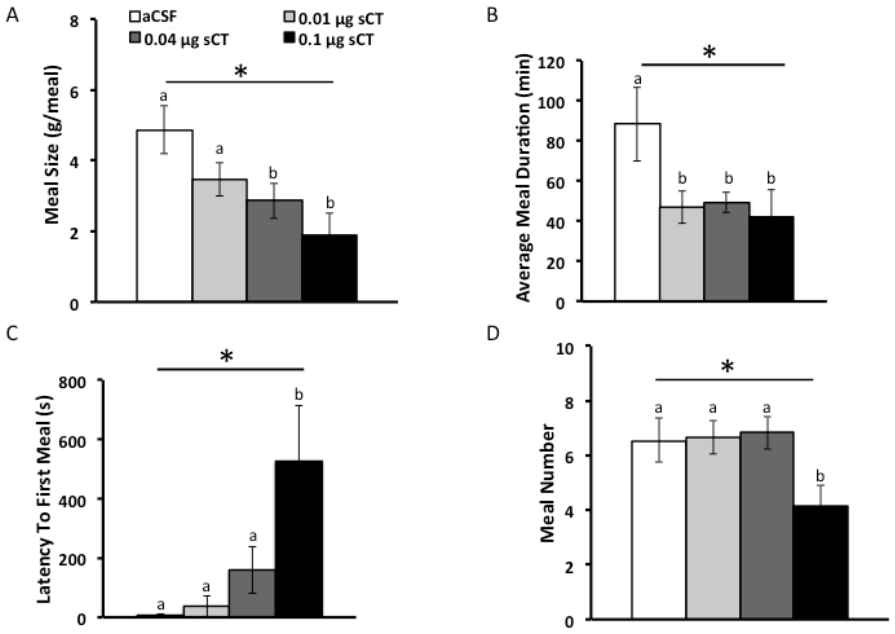

Figure 3. Intra-LDTg amylin receptor activation predominately suppresses meal size rather than meal frequency. To determine the behavioral mechanism driving intake suppression, animals were housed in a custom-made automated feedometer to analyze meal patterns. The amylin receptor agonist, sCT, was unilaterally injected into the LDTg in a counterbalanced within subjects design at the onset of the dark cycle using the following doses: 0 (aCSF), 0.01, 0.04, and 0.1 µg (n=5). Intra-LDTg sCT suppresses meal size over 24h at the two higher doses (A), but all 3 doses suppress average meal duration over 24h (B). Only the highest dose of sCT increases latency to first meal (C) and suppresses meal frequency over 24h (D). The key applies to all graphs. * indicates significance by repeated measures ANOVA ($p<0.05$), different letters are significantly different from each other according to post-hoc tests ($p<0.05$).

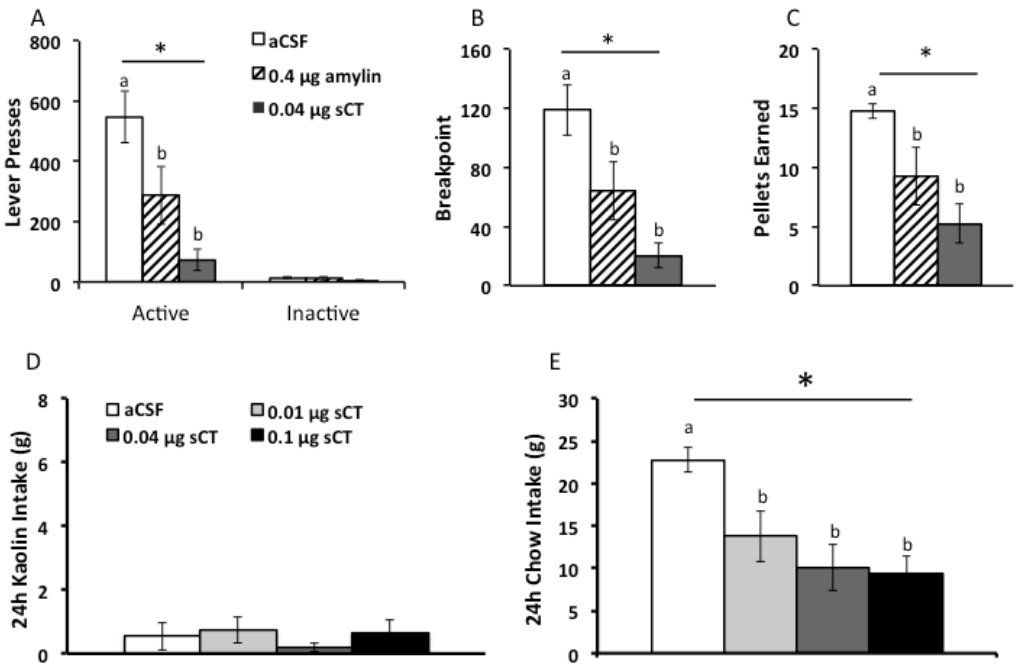

Figure 4. Intra-LDTg amylin receptor activation suppresses motivated feeding but does not produce malaise. The ability of LDTg amylin receptor activation to reduce sucrose self-administration on a PR schedule of reinforcement was assessed (n=8). Intra-LDTg amylin receptor activation with amylin (0.4µg) or sCT (0.04µg) suppresses active lever presses (A), breakpoint (B), and pellets earned (C). To determine if LDTg amylin receptor activation produces nausea/malaise, pica (ingestion of non-nutritive substances in response to a noxious stimulus) was measured. Animals received access to both chow and kaolin clay for one week prior to the beginning of the experiment. The amylin receptor agonist, sCT, was unilaterally injected into the LDTg using the following doses: 0 (aCSF), 0.01, 0.04, and 0.1 µg (n=6). Intra-LDTg amylin receptor activation does not increase kaolin clay intake (D) but suppresses chow intake at 24h (E). Key in A applies to A, B and C; key in D applies to D and E. * indicates significance by repeated measures ANOVA ($p<0.01$); different letters are significantly different from each other according to post-hoc tests ($p<0.05$).

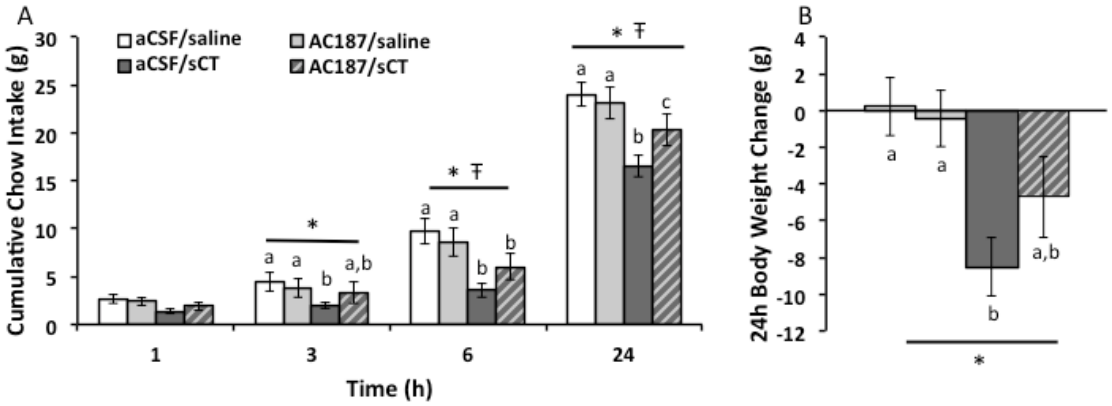

Figure 5. LDTg amylin receptor blockade attenuates the intake suppressive effects of an amylin receptor agonist. To determine if LDTg amylin receptor signaling is pre-clinically relevant, the amylin receptor antagonist, AC187, was bilaterally injected in the LDTg (0.8µg/hemisphere) followed 45 minutes later by a systemic injection of sCT (5µg/kg, IP) shortly before the onset of the dark cycle (n=11). Pre-treatment of AC187 alone has no significant effect on chow intake or body weight at any time point. Administration of sCT significantly suppresses intake at 3, 6, and 24h (A) as well as 24h body weight gain (B). Pre-treatment of AC187 with sCT significantly attenuates the intake suppressive effects of systemically-delivered sCT. Legend applies to both graphs. * indicates a significant main effect of sCT by repeated measures ANOVA ($p<0.01$), ∓ indicates a significant main interaction between sCT and AC187 by repeated measures ANOVA ($p<0.05$), and different letters are significantly different from each other according to post-hoc planned comparisons ($p<0.05$).

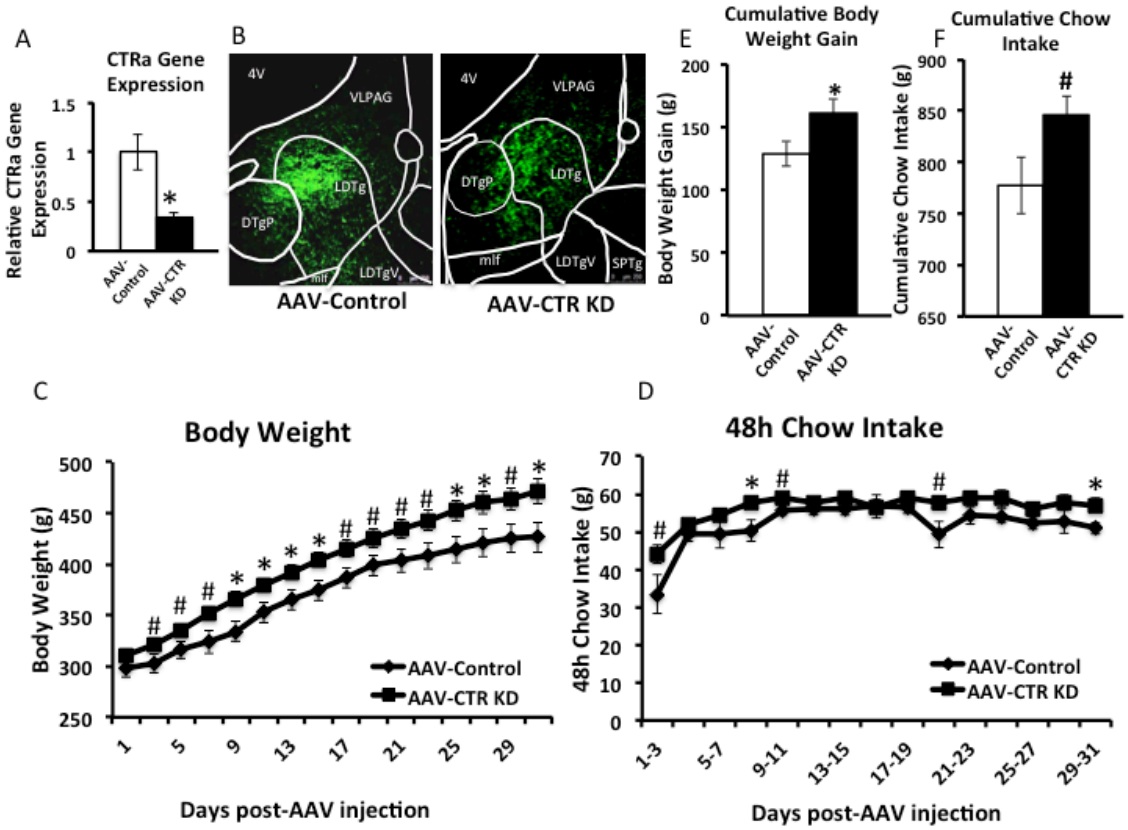

Figure 6. CTR knockdown in the LDTg produces sustained increases in body weight and chow intake. To determine if LDTg amylin receptor signaling is physiologically relevant for the long-term control of food intake and body weight regulation, an AAV that knocks down the core component of the amylin receptor, the CTR (AAV-CTR KD), or an empty vector AAV (AAV-Control) was injected bilaterally in the LDTg (200nl/hemisphere). Food intake and body weight was measured every 48h for 31 days following viral injection (n=7/viral condition). (A) Compared to AAV-Control, the AAV-CTR KD produces a statistically significant 67% decrease of CTRa. A separate cohort of animals received either virus (n=3/viral condition), were sacrificed two weeks later, and the brains were processed for GFP visualization. Representative images show GFP labeling of viral expression in AAV-Control (left) and AAV-CTR KD (right) (B). In behavioral studies, AAV-CTR KD produces an increase in body weight that was sustained over the behavioral test period (C, E). Chow intake is transiently increased in AAV-CTR KD animals compared to AAV-Control animals when graphed in 48 bins (D), and trending for significance when graphed cumulatively over the entire behavioral test period (F, $p<0.1$). * indicates significance by ANOVA ($p \leq 0.050$), # indicates a trend for significance by ANOVA ($p<0.1$). 4V = 4th ventricle, DTPg = Dorsal tegmental nucleus pericent, LDTg= lateral dorsal tegmental nucleus, LDTgV = lateral dorsal tegmental nucleus ventral, mlf = medial longitudinal fasciculus, SPTg = subpeducuncular tegmental nucleus, VLPAG = ventral lateral periaqueductal gray.

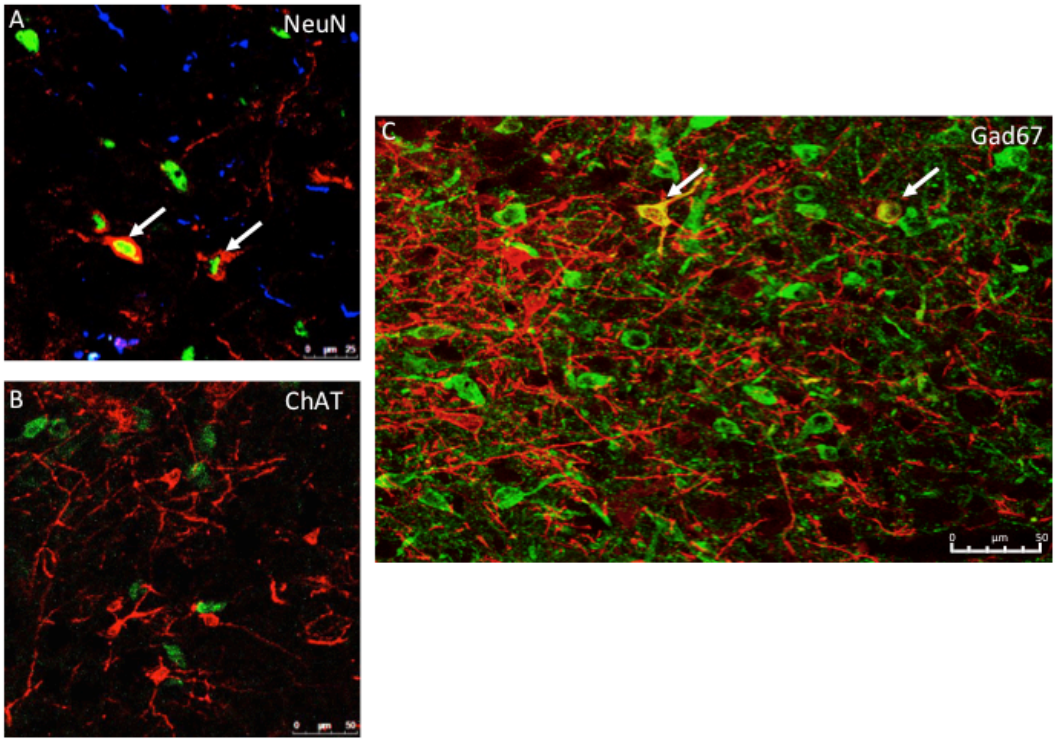

Figure 7. CTR-expressing cells in the LDTg are GABAergic. IHC analyses show that CTR-expressing cells in the LDTg co-localize with the neuronal marker NeuN and not with the glial cell marker GFAP (A; 20x with a 2x optical zoom; n=3). CTR-expressing cells in the LDTg do not co-localize with the cholinergic marker ChAT (B; 40x; n=6) but co-localize with the GABAergic marker Gad67 (C; 20x; n=1). Red = CTR-positive cells; Blue = GFAP-positive cells; Green = cellular marker of interest: NeuN (A), ChAT (B), Gad67 (C). White arrows indicate co-localization.

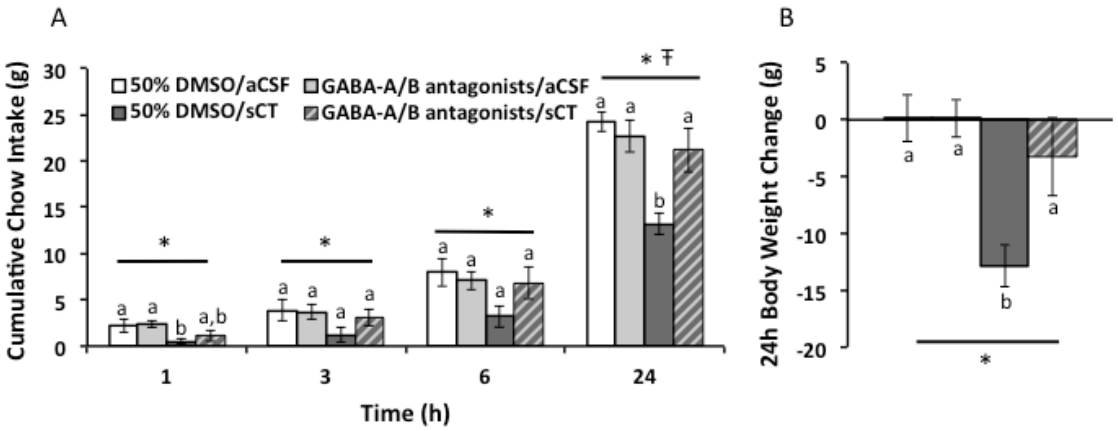

Figure 8. Intra-LDTg GABA receptor blockade reverses the intake suppressive effects of intra-LDTg amylin receptor activation. To determine the role of GABA receptor signaling in the intake suppressive effects of LDTg amylin receptor activation, a cocktail of a GABA-A receptor antagonist (bicuculline, 100 ng) and a GABA-B receptor antagonist (saclofen, 500 ng) was administered unilaterally in the LDTg followed by sCT (0.04µg; 100 nl; n=8). GABA receptor blockade reverses the intake (A) and body weight-suppressive effects (B). Key applies to both graphs. * indicates a significant main effect of sCT (A) or treatment (B) by repeated measures ANOVA ($p<0.05$), Ŧ indicates a significant main interaction between sCT and the GABA-receptor antagonists by repeated measures ANOVA ($p<0.05$), different letters are significantly different from each other according to post-hoc planned comparisons ($p<0.05$).

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

There is a critical need for basic science research to identify key neurochemical systems that can be targeted by current and future pharmacotherapies aimed at treating obesity and its associated metabolic co-morbidities. To this end, identifying the site(s) of action (e.g. cellular and nuclei specificity) of endogenous energy balance signals and long-acting clinically relevant receptor agonists is paramount in treating human obesity. The data presented in this dissertation provide evidence that GLP-1Rs expressed on astrocytes in the NTS are relevant for energy balance control and that GLP-1 and amylin individually act in the LDTg to control energy balance. As a whole, these data broaden the view of CNS actions of GLP-1 and amylin, in terms of both the cellular and regional control of energy balance. In this chapter, I summarize key findings, discuss questions these data raise, and propose future avenues of research.

What is the purpose of astrocytic expression of GLP-1R?

Data presented in Chapter 2 show that NTS astrocytes express GLP-1Rs, respond appropriately to GLP-1R activation with increases in intracellular calcium and cAMP, and are behaviorally relevant for energy balance control. These findings are part of a growing body of literature showing that astrocytes respond to energy status signals and contribute to energy balance control [see (Kim et al., 2014; Wang et al., 2015; Frago and Chowen, 2017) for review]. These data establish a novel role for NTS astrocytes in GLP-1R signaling and energy balance control more broadly, but the biological function of NTS astrocytes needs to be investigated.

These data raise the natural question of the biological/physiological purpose of GLP-1R signaling in NTS astrocytes in coordinating energy balance control. As

presented in Chapter 2, one potential source of GLP-1 to NTS astrocytes is from PPG neurons within the NTS, indicated by triple labeling of GLP-1, GFAP, and synaptophysin. It is likely that astrocytes form a tripartite synapse with NTS neurons, though the precise structure and dynamics of this putative neuron-glia interaction for GLP-1R signaling needs to be further investigated. Future research should include ultrastructure analyses with electron microscopy to examine the putative tripartite synapse between neurons and GLP-1R-expressing astrocytes. Following GLP-1R activation, research has shown increases in intracellular calcium and cAMP, PKA and MAPK activation, and downregulation of AMPK (Perfetti and Merkel, 2000; Gomez et al., 2002; Hayes et al., 2011b). However, when studies using tissue lysates to examine GLP-1R signaling results cannot distinguish between cell types. While data presented in Chapter 2 show increases in intracellular calcium in both astrocytes and neurons following bath application of Ex-4, the magnitude of response is greater in astrocytes than neurons, suggesting that there may be unexplored cell type-dependent differences in downstream cell signaling cascades. It is therefore critically important to first determine the separate intracellular signaling cascades after GLP-1R activation in astrocytes versus neurons (e.g. with cultured astrocytes and cultured neurons) and then to determine whether the responses vary when astrocytes and neurons are expressed together (e.g. in co-cultures of both cell types). As modern techniques allow for visualization of calcium dynamics *in vivo* in awake behaving animals (Akerboom et al., 2012; Gee et al., 2015; Srinivasan et al., 2016), future experiments could measure calcium in NTS astrocytes and neurons from awake, behaving animals in response to a variety of stimuli (daily meals, systemic or central GLP-1R activation, exogenous administration of energy status signals, gastric distension, etc.). Furthermore, this technique has the potential to provide a holistic view

of the role of astrocytes in energy balance control, by allowing for the analysis of calcium dynamics within astrocytes throughout the CNS after administration of energy status signals (e.g. GLP-1, amylin, leptin, ghrelin, etc.), with diet manipulations, or as a function of state (e.g. fed vs fasted, lean vs obese). Collectively, data in Chapter 2 establish a role for NTS astrocytes in GLP-1-mediated energy balance control, suggesting that astrocytes likely interact with neurons as part of a tripartite synapse in the NTS to respond to the environment and contribute to energy balance control.

Given that a predominant role of astrocytes within a tripartite synapse is the regulation of synaptic glutamate levels (Perego et al., 2000; Rimmele and Rosenberg, 2016), could GLP-1R-expressing NTS astrocytes modulate glutamate levels and if so, how might that affect food intake? As part of the canonical signaling pathway following GLP-1R activation, PKA levels increase (Hayes et al., 2011b; Rupprecht et al., 2013), and separately, PKA can modulate expression of glutamate transporters (Lim et al., 2005). We hypothesize that GLP-1R activation in NTS astrocytes decreases glutamate transporter expression, which would thereby increase the level of synaptic and extrasynaptic glutamate. Elevated glutamate levels would then putatively lead to downstream activation of AMPA and NMDA receptors, which have been shown to mediate GLP-1R-dependent suppression of food intake (Hisadome et al., 2011; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2014). Therefore, the expression of NTS glutamate transporters (GLT-1 and GLAST) should be evaluated in a time-specific manner following GLP-1R activation, and the role of potential changes in glutamate transporter expression in GLP-1R-mediated suppression of food intake should be functionally tested (e.g. with ceftriaxone, a GLT-1 upregulator). If our hypothesis is correct and GLP-1R activation decreases glutamate transporter expression,

administration of ceftriaxone should attenuate or reverse GLP-1-mediated hypophagia by increasing GLT-1 expression.

In addition to modulating synaptic glutamatergic signaling, following chemical activation and through a calcium-dependent process, astrocytes within a tripartite synapse can release gliotransmitters (Nedergaard et al., 2003; Ransom et al., 2003; Halassa et al., 2007; Barres, 2008; Eroglu and Barres, 2010; Clarke and Barres, 2013; Robinson and Jackson, 2016). Given that the live cell calcium imaging data in Chapter 2 show a robust increase in intracellular calcium in NTS astrocytes following bath application of Ex-4, it is possible that GLP-1R mediated activation of astrocytes may stimulate the release of gliotransmitters. The role of gliotransmission should therefore be functionally tested with pharmacology, for example by probing the response to GLP-1R activation in an astrocyte-specific dominant negative Soluble N-ethylmaleimide-sensitive factor activation protein receptor (SNARE) animal, in which gliotransmission is genetically prevented. Collectively, data presented in Chapter 2 provides evidence in support of the importance of NTS astrocytes in GLP-1R-mediated energy balance control, and also sets the foundation for further investigation of the functional role of NTS astrocytes in modulating neuronal activity to control energy balance.

Data presented in Chapter 2 showing that NTS astrocytes can bind peripherally administered GLP-1R agonists is consistent with the idea that astrocytes can sense blood-borne factors (Yi et al., 2011; Chowen et al., 2016; Leloup et al., 2016; Argente-Arizon et al., 2017), These data support a growing body of evidence that the NTS is a critical site in mediating GLP-1R activation and provide further evidence that this nucleus is potentially clinically relevant for GLP-1R-mediated energy balance control (Hayes et

al., 2008; Grill and Hayes, 2009; Hayes et al., 2011a; Hayes et al., 2011b; Grill and Hayes, 2012; Rupprecht et al., 2013; Alhadeff and Grill, 2014; Alhadeff et al., 2016). In addition to sensing blood-borne factors, astrocytes can modulate the permeability of the blood brain barrier (BBB) through associations with endothelial cells and release of chemical factors (Abbott et al., 2006). One intriguing avenue of research would be to examine astrocytic modulation of BBB permeability and determine whether CNS penetrance of neuroendocrine signals (e.g. GLP-1, amylin) changes based on state of the animal (fed vs. fasted, lean vs. obese, male vs. female). If BBB permeability deteriorates in the obese state, leading to altered endogenous gut-brain signaling, could improved constructs of neuroendocrine receptor agonists result in increased BBB penetrance and normalize central energy balance control? Alternatively, would targeting astrocytes/endothelial cells improve BBB penetrance and lead to a restoration of endogenous gut-brain signaling in the obese state? Collectively, the data presented in Chapter 2 invoke these important and clinically-relevant questions regarding BBB penetrance of energy status signals and how astrocytes might be therapeutic targets to modulate BBB permeability.

Importantly, NTS astrocytic expression of GLP-1R raises the question of whether GLP-1R are expressed on astrocytes in other nuclei important for energy balance and reward. For example, data in Chapter 3 shows that fluorescent exendin-4 associates with predominately neurons and minimally with astrocytes in the LDTg, contrary to the findings in Chapter 2, which show internalization in both cell types in the NTS. These data in the NTS and LDTg raise the question of how cell type-specific expression of GLP-1R changes depending on the nuclei examined. The GLP-1 field is stymied by the lack of a commercially available GLP-1R-specific antibody. Therefore, there is great

utility for the commercially available fluorescently-labeled exendin-4 used in these studies to label GLP-1R-expressing cells, which will allow for determination of cell-type expression of GLP-1R in nuclei throughout the brain. Having this proxy to identify GLP-1R-expressing cells in the rat and across species should be an advantageous tool for many researchers in the field and is already being used by other laboratories (Terrill et al., 2016).

The data in Chapter 2 show fluorescent exendin-4 labeling within cells, suggesting that the exendin-4 and theoretically the GLP-1R are internalized in the NTS at 3h post-injection. To our knowledge, we are the first to provide *in vivo* evidence of GLP-1R internalization and our data are in agreement with *in vitro* data showing GLP-1R are internalized following activation *in vitro* (Roed et al., 2014; Shaaban et al., 2016). *In vitro* data show that the GLP-1R is internalized shortly after exposure to GLP-1 or GLP-1R agonists (e.g. minutes), and eventually is recycled back to the cell membrane (takes 2-3 times longer for exendin-4 and liraglutide compared to GLP-1) (Roed et al., 2014). However, the purpose of this internalization in neural tissue or brain-derived primary cell cultures has not been established. The use of fluorescent GLP-1R agonists provides the exciting possibility to visualize and track GLP-1 binding, activation, and internalization in real time with live cell imaging in *in vitro* and potentially *ex vivo* preparations in order to examine cell-type differences in the dynamics of cellular activation of GLP-1Rs.

While data presented in Chapter 2 suggest that GLP-1Rs are expressed on NTS astrocytes in the rat, data in the literature has revealed an absence of astrocytic expression of GLP-1R in the mouse (Cork et al., 2015). These data are part of a small body of literature describing species differences in the GLP-1 system between mice,

rats, non-human primates, and humans (Lachey et al., 2005; Vrang and Grove, 2011; Cork et al., 2015; Heppner et al., 2015). The biology and physiology of the GLP-1 system in rats seems to mimic that of non-human primates and humans more closely than in mice, making rats the appropriate animal model for this dissertation (Lachey et al., 2005; Vrang and Grove, 2011; Cork et al., 2015; Heppner et al., 2015). Given these data, astrocytic GLP-1R expression might extend to higher order species beyond the rat, but this should be empirically tested.

Collectively, data presented in Chapter 2 establish novel cell-type dependent effects for GLP-1R activation in modulating energy balance and emphasize the importance of studying the contribution of astrocytes to energy balance control. This underscores the need to evaluate novel targets for GLP-1R agonists that may be valuable for treating obesity. In Chapters 3 and 4, the idea of examining novel potential targets for obesity pharmacotherapies was brought to the level of the CNS nucleus, and examined the relevance of the LDTg for energy balance control.

The LDTg: an understudied hub in energy balance regulation

Data presented in Chapters 3 and 4 demonstrate that the LDTg responds to both GLP-1 and amylin to control food intake and body weight. In Chapter 3, pharmacological activation of LDTg GLP-1Rs decreases food intake and body weight, primarily through a reduction in meal size and independent of nausea/malaise. Pharmacological blockade of LDTg GLP-1Rs increases food intake, and a subthreshold dose of the GLP-1R antagonist exendin-9 administered in the LDTg attenuates the intake suppressive effects of peripheral exendin-4 or of a gastric pre-load. IHC data show the presence of GLP-1-immunopositive fibers in the LDTg and that NTS PPG neurons project to the LDTg,

providing anatomical evidence that GLP-1R signaling in the LDTg in physiologically relevant. Furthermore, a peripherally administered fluorescently-labeled GLP-1R agonist can be visualized within the LDTg and is juxtaposed to neurons and minimally to astrocytes. Together, these data show that GLP-1R signaling in the LDTg contributes to energy balance control.

Data presented in Chapter 4 examine another neuroendocrine system that is potentially clinically relevant for the treatment of obesity, amylin. Findings show that pharmacological activation of LDTg amylin receptors decreases food intake and body weight, primarily through a reduction in meal size and independent of nausea/malaise. Blockade of LDTg amylin receptors attenuates the intake suppressive effects of peripheral amylin receptor activation, while chronic knockdown of LDTg amylin receptors increases food intake and body weight. Furthermore, a portion of LDTg amylin receptors is GABAergic, and GABA receptor signaling in the LDTg is required for the hypophagic effects of LDTg amylin receptor activation. Together, these data show that amylin receptor signaling in the LDTg contributes to energy balance control.

Collectively, data in Chapters 3 and 4 support a role for the LDTg in processing neuroendocrine signals to control energy balance. These data are part of a growing body of literature showing that the LDTg responds to neuroendocrine signals to control energy balance. For example, ghrelin receptors are expressed in the LDTg, and ghrelin administration in the LDTg increases food intake via elevated acetylcholine release in the VTA and dopamine release in the NAc (Jerlhag et al., 2007; Dickson et al., 2010; Dickson et al., 2011; Jerlhag et al., 2012). The LDTg is clearly an energy balance-relevant nucleus that is just beginning to be explored, though to date and including this

dissertation, studies have examined individual neuroendocrine signals within the LDTg for energy balance control. It is important to consider that the endogenous response to a meal includes the release of many neuroendocrine signals both from peripheral and central sources which can, in theory, interact in the LDTg to control energy balance. Indeed, the LDTg expresses a variety of receptors for neuroendocrine signals including, GLP-1, amylin, ghrelin, oxytocin, orexin, and PYY, and thus may integrate these signals to modulate energy balance (Sexton et al., 1994; Merchenthaler et al., 1999; Parker and Herzog, 1999; Greco and Shiromani, 2001; Gould and Zingg, 2003; Dickson et al., 2011; Cabral et al., 2013).

Data in the literature support additive/synergistic effects on food intake with combination therapy of GLP-1 and amylin-based drugs (Bello et al., 2010). As data presented in this dissertation establishes a role for LDTg GLP-1R and separately LDTg amylin signaling for energy balance control, further investigation is needed to examine the potential interaction between GLP-1R signaling and amylin receptor signaling in the LDTg. One hypothesis is that LDTg amylin receptor signaling interacts with gut-brain signaling through a NTS-LDTg GLP-1 pathway. To support this hypothesis, anatomical data would need to show that NTS-originating GLP-1 fibers are in close apposition to LDTg CTR-expressing neurons and that CTR-expressing cells within the LDTg bind fluorescent Ex-4. These data would provide anatomical evidence that GLP-1R activation can enhance the effects of amylin receptor activation, potentially by acting on the same cell. Behavioral evidence would support this hypothesis if LDTg GLP-1R blockade attenuates the intake suppressive effects of LDTg amylin receptor activation.

In addition, the temporal dynamics of LDTg GLP-1 and amylin-mediated hypophagia should be explored. Data presented in Chapter 3 shows that a suppression in food intake is not observed until 6h after intra-LDTg Ex-4 treatment, but data presented in Chapter 4 shows that LDTg amylin receptor activation decreases food intake as early as 1h after injection. This difference in timing of intake suppression has also been observed in the VTA (Alhadeff et al., 2012; Mietlicki-Baase et al., 2013a; Mietlicki-Baase et al., 2013b; Mietlicki-Baase et al., 2015b). It is therefore important to examine the downstream effects of LDTg GLP-1R and amylin receptor activation and why temporal differences in feeding effects are observed. Are there differences in intracellular signaling pathways or projection patterns that would result in a slower onset of behavioral effects with centrally-administered Ex-4 compared to sCT or amylin?

The interaction between orexigenic hormones (e.g. ghrelin and orexin) and anorexigenic hormones (e.g. GLP-1 and amylin) at the level of the LDTg remains unexplored. Though this dissertation focuses on two within-meal satiation signals in the LDTg, it is possible that the LDTg integrates pre-prandial and post-prandial release of energy status signals to control feeding. The precise LDTg cell types that express receptors for these hormones and the dynamics of how these hormones interact in the LDTg to potentially modulate energy balance should be explored.

A growing body of literature shows that the LDTg modulates VTA dopamine cell firing and dopamine release in the nucleus accumbens, which ultimately mediates motivated behavior, including food intake as well as reward behaviors and drug seeking (Omelchenko and Sesack, 2005; Lodge and Grace, 2006; Shabani et al., 2010; Lammel et al., 2012; Shinohara et al., 2014; Steidl et al., 2015; Schmidt et al., 2016; Steidl et al.,

2017). While data presented in Chapter 3 show that peripherally-administered fluorescent Ex-4 is juxtaposed with neurons more so than astrocytes, these GLP-1R-expressing LDTg neurons need to be further characterized in terms of their neurochemical phenotype and projection patterns. Given that a portion of LDTg amylin receptor-expressing neurons are GABAergic, it is possible that LDTg amylin receptor activation inhibits VTA dopamine neurons either through local inhibition (e.g. as an interneuron) of VTA-projecting cholinergic/glutamatergic LDTg neurons or through direct inhibition (e.g. as a projection neuron) of VTA dopamine neurons. Alternatively or additionally, amylin receptor expressing LDTg neurons may modulate other nuclei to control feeding, such as the lateral habenula or dorsal raphe, which have been associated with negative valence and avoidance/aversion (Matsumoto and Hikosaka, 2009; Li et al., 2013). Neural pathway tracing coupled with immunohistochemistry will begin to elucidate these possibilities, both in terms of the projection patterns of GLP-1R and amylin receptor-expressing neurons and if GLP-1R are expressed on amylin-receptor expressing neurons. Given the close overlap in the circuitry underlying feeding and addiction (Narayanan et al., 2010; DiLeone et al., 2012), these neuroanatomical data could have important implications not only for the field of energy balance and obesity, but also for the fields of motivated behavior and drug addiction.

In addition to effects on energy balance, GLP-1 has been shown to modulate other rewarding behaviors such as drug taking and seeking (Graham et al., 2013; Sorensen et al., 2015; Schmidt et al., 2016; Vallof et al., 2016). Though less commonly studied in the drug abuse field, amylin has been shown to interact with the endogenous opioid system and modulates the dopamine system (Clapper et al., 2013; Mietlicki-Baase and Hayes, 2014; Mietlicki-Baase et al., 2015b). Given these findings, and that

GLP-1 and amylin mimetics are FDA approved for diabetes and/or obesity treatment, it is possible that these analogs could be repurposed to treat drug addiction. Indeed, in addition to effects on drug taking (Schmidt et al., 2016), exciting data from the Schmidt lab show that central administration of Ex-4 and separately amylin attenuates cocaine reinstatement, an animal model of relapse (unpublished studies). Future research in animal models of drug addiction and drug relapse should therefore evaluate the utility of GLP-1 and amylin-based pharmacotherapies for treating addiction across drug classes and elucidate and the CNS mechanisms by which they act.

One critical question raised by the data presented in Chapters 3 and 4 is how does the LDTg putatively integrate endogenous GLP-1R and amylin receptor signaling to control energy balance. One possible hypothesis is that a portion of LDTg neurons express both the GLP-1R and the amylin receptor and can be activated by both neuroendocrine signals. In this case, the onset of a meal would result in increased secretion of amylin by the pancreas, elevated plasma levels of amylin, and ultimately amylin receptor activation in the CNS, including the LDTg. As the meal continues and the stomach becomes further distended, NTS PPG neurons would be activated and theoretically increase synaptic release of GLP-1 throughout the CNS, including in the LDTg. Thus, LDTg levels of amylin and GLP-1 would increase throughout the meal and activate LDTg neurons. However, in this scenario, there is likely a short delay (e.g. minutes during the meal) between when endogenous amylin and endogenous GLP-1 accesses the LDTg, such that amylin may access the CNS first and activate LDTg amylin-receptor expressing neurons. LDTg neurons that express both GLP-1Rs and amylin receptors would be activated by both neuroendocrine signals, leading to sustained excitation of these neurons that perhaps are GABA neurons that project to the

VTA to modulate dopamine cell firing. Sustained excitation of these neurons would lead to an amplification of within meal satiation signaling, a decrease in the rewarding value of the meal, and ultimately promote for meal termination. It is also possible that there is also a subset of LDTg neurons that express GLP-1Rs but not amylin receptors and a subset of LDTg neurons that express amylin receptors but not GLP-1Rs. In addition, energy balance control is distributed throughout the CNS and these putative actions of GLP-1 and amylin within the LDTg are only representative of how one CNS nucleus integrates neuroendocrine signals to control energy balance.

CONCLUSION

Collectively, the data presented in this dissertation broaden the perspective on the specific cell types and brain regions that respond to neuroendocrine signals and control energy balance. These data, from normal or lean male rats, provide critical foundational evidence for understanding the biology and physiology of the CNS and gut-brain signaling, and also provide important knowledge for improving pharmacological tools to treat obesity. The ultimate goal of this information is to understand how these systems might be dysregulated in the obese state and how targeting multiple systems in a combination therapy of drugs may produce a greater magnitude of weight loss than individual monotherapies. The specific combination of GLP-1 and amylin-based drugs for obesity treatment has received attention of the past several years (Bello et al., 2010; Roth et al., 2012; Jorsal et al., 2016; Gydesen et al., 2017) and data presented in this dissertation emphasize the importance of examining both the local (cell-type) and global (brain-wide) effects of GLP-1 and amylin administration for potential additivity or synergy in decreasing food intake and promoting weight loss.

APPENDIX

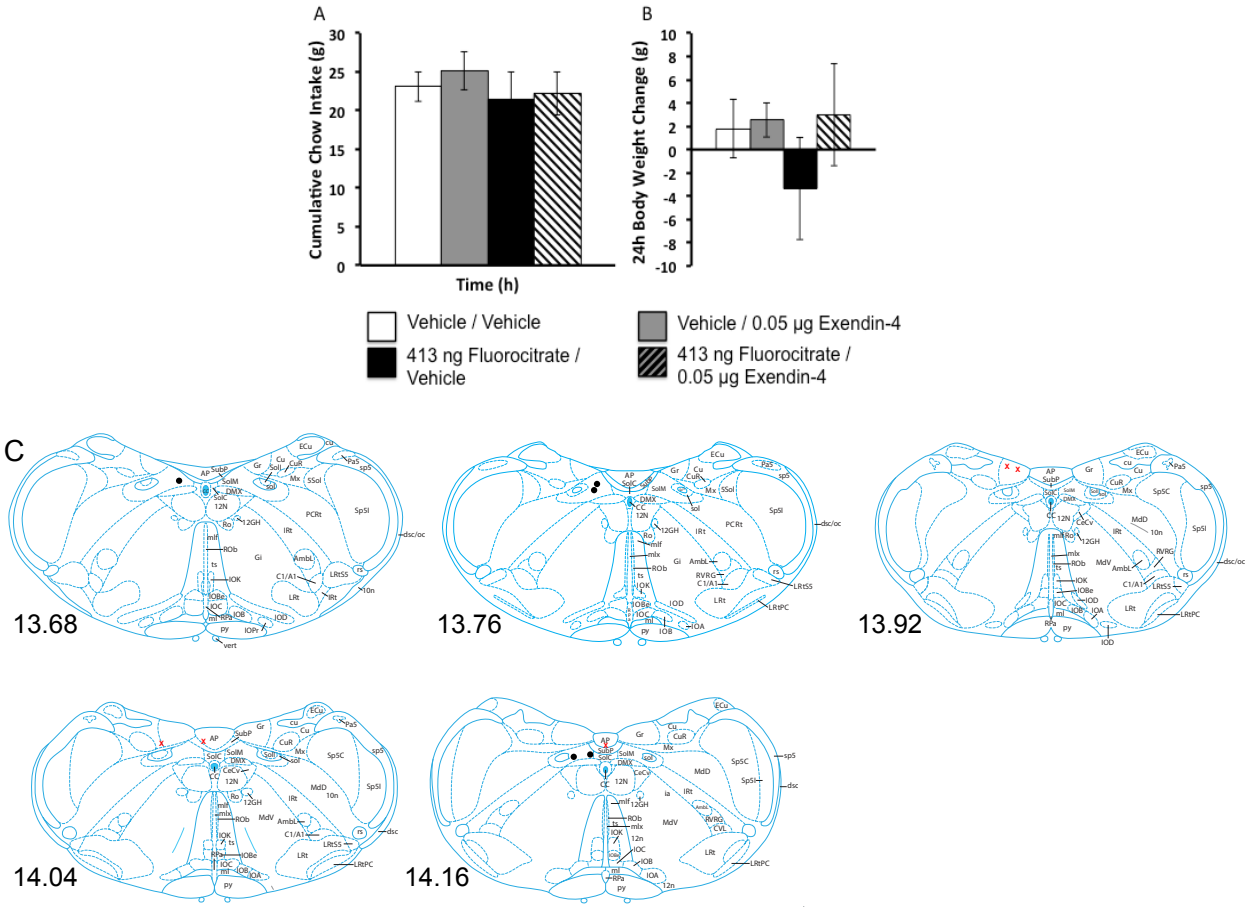

Figure A1. Intra-NTS GLP-1R activation in animals with missed cannula placements does not produce hypophagia. Rats (n=5) were given an intra-mNTS pretreatment with the astrocyte Krebs cycle inhibitor fluorocitrate or its vehicle, followed by direct mNTS administration of the GLP-1R agonist Ex-4. There is no significant main effect of either drug on food intake (A) or body weight (B) ($F_{1,4}$<3.6, p>0.13). Data shown as mean ± SEM. Cannula placements are shown in C with black dots indicating cannula placements that accurately targeted the mNTS (SolM in C) and behavioral data from these animals is graphed in Figure 2.7. Red X's indicate cannula placements that missed the mNTS and behavioral data from these animals is graphed here in Figure A1A-B. Numbers represent distance (mm) from bregma according to Paxinos & Watson (2007). AP = area postrema, Gr = nucleus gracilis, SolM = medial nucleus tractus solitarius.

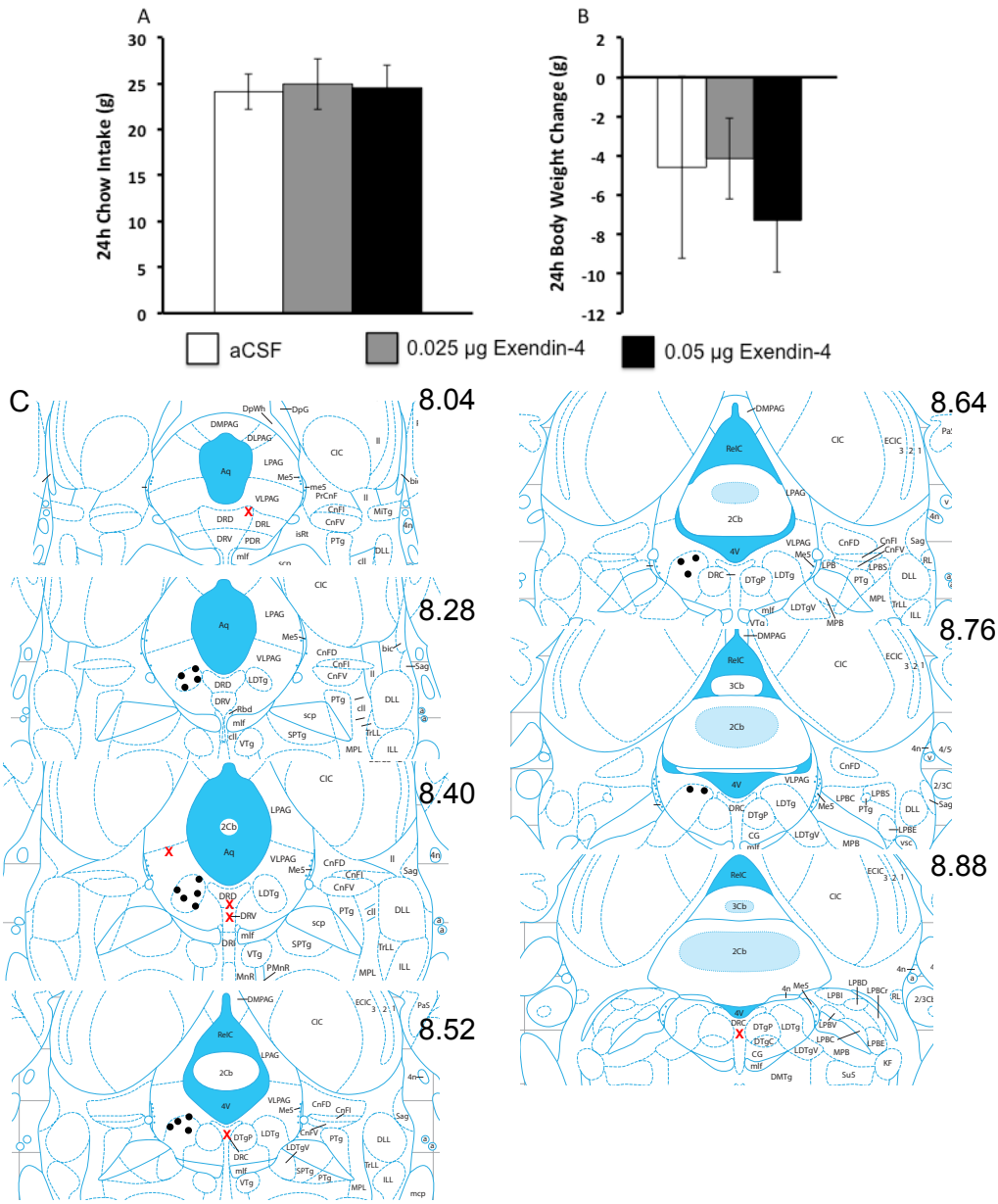

Figure A2. Intra-LDTg GLP-1R activation in animals with missed cannula placements does not produce hypophagia. The GLP-1R agonist Ex-4 was unilaterally injected in the LDTg in a counterbalanced within-subjects design at the onset of the dark cycle (n=6). There is no significant main effect of drug on food intake (A) or body weight (B) ($F_{1,3}$<0.23, p>0.80). Data shown as mean ± SEM. Cannula placements are shown in C with black dots indicating cannula placements that accurately targeted the LDTg and behavioral data from these animals is graphed in Figure 3.1C-F. Red X's indicate cannula placements that missed the LDTg and behavioral data from these animals is graphed here in Figure A2A-B. Numbers represent distance (mm) from bregma according to Paxinos & Watson (2007). DRC = dorsal raphe caudal, DRD = dorsal raphe dorsal, LDTg = lateral dorsal tegmental nucleus, VLPAG = ventrolateral periaqueductal gray.

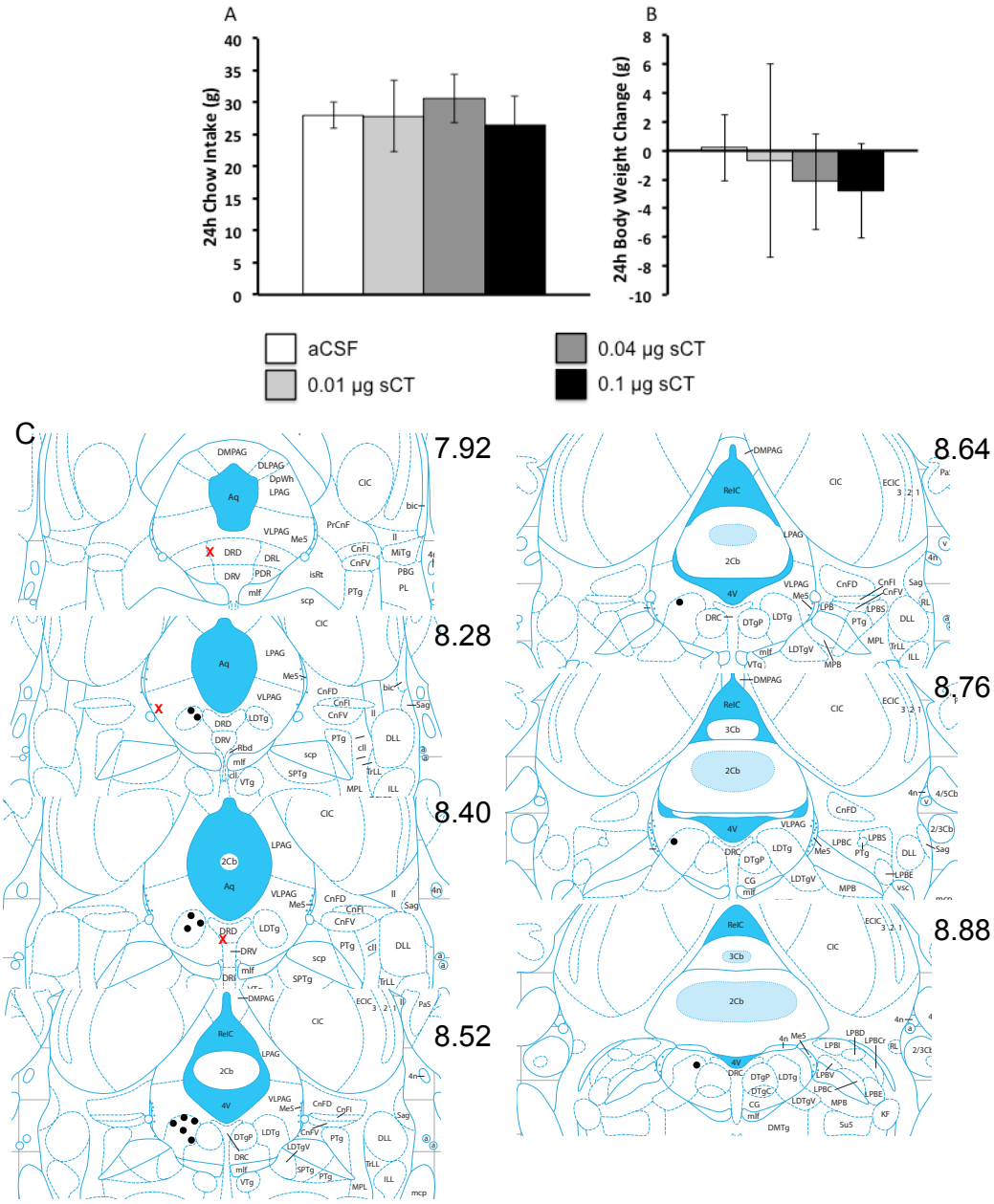

Figure A3. **Intra-LDTg amylin receptor activation in animals with missed LDTg cannula placements does not cause hypophagia.** The amylin receptor agonist sCT was unilaterally injected into the LDTg in a counterbalanced within subjects design at the onset of the dark cycle (n=3). There is no significant effect on food intake (A) or body weight (B). ($F_{1,3}$<0.6, p>0.64). Data shown as mean ± SEM. Cannula placements are shown in C with black dots indicating cannula placements that accurately targeted the LDTg and behavioral data from these animals is graphed in Figure 4.3 and 4.4C-D. Red X's indicate cannula placements that missed the LDTg and behavioral data from these animals is graphed here in Figure A3A-B. Numbers represent distance (mm) from bregma according to Paxinos & Watson (2007). DRD = dorsal raphe dorsal, LDTg = lateral dorsal tegmental nucleus, VLPAG = ventrolateral periaqueductal gray.

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