HINDBRAIN NEURAL TRACKING AND CONTROL OF FOOD INTAKE

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Food intake is the product of meal size and frequency. Meal size is determined by sensory feedback signals that arise from the gastrointestinal (GI) tract and other digestive organs during feeding. Hindbrain glucagon-like peptide-1 (GLP-1) and prolactin-releasing peptide-positive noradrenergic A2 neurons (PrRP-positive) are anatomically poised to receive multimodal ingestive/metabolic-related information and engage local oral motor circuits that govern feeding. Moreover, multiple lines of evidence support a role for signaling from these populations in the suppression of food intake, while a role in the day-to-day control of meal size is less clear. Considering this, we hypothesized that GLP-1 and PrRP A2 neurons participate in satiation.

Results from studies conducted herein demonstrated that the proportion of GLP-1 and A2 neurons that were activated by food intake closely reflects both the volume and caloric value of the food consumed, and this activation was modulated by experience with consuming unusually large meals. Among GLP-1 neurons, those in the caudal nucleus of the solitary tract (cNTS) were more sensitive than those in the neighboring medullary reticular formation (MRF), supporting direct vagal afferents as an important route by which sensory information recruits

these neurons. PrRP-positive A2 neurons were more sensitive than PrRP-negative A2 neurons to feeding-related sensory signals, suggesting that PrRP may be a particularly important among the many neuropeptide products of A2 neurons in the context of feeding control. In Chapter 4, we demonstrated that blocking central GLP-1 receptors produced larger and longer meals consumed during the latter phases of the feeding period, without changing meal frequency or the interval between meals. These results indicated a causal role for central GLP-1 signaling in satiation specifically.

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PREFACE

Neither this dissertation nor my development as a young scientist would have been possible without the support of many people. Most of all, I would like to thank my mentor Linda Rinaman for continually challenging me to think, write, and conduct experiments like a top-notch scientist. I would also like to extend the deepest heartfelt thank you to my former lab mate Dr. James Maniscalco for being the best graduate student role model and friend I could have asked for during my first 4 years. Thank you to Vicki Maldovan, Li Cai, and Huiyuan Zheng for their technical support throughout my graduate career and to my undergraduate mentees Elizabeth Davis and Mitchell Bayne for their technical contributions and enthusiasm. I would also like to thank Joan Blaney, Patti Argenzio and Marlene Nieri for being so reliable and supportive when it came to administrative manners. Thank you to my husband Evan for his continued support and acceptance despite not always understanding this whole graduate school thing. Lastly, thank you to my parents, Nancy and Fritz, for their undying interest in my graduate endeavors, their mentorship and for instilling in me an interest in scientific pursuit and psychology/biology since as early as I can remember.

LIST OF ABBREVIATIONS

ANGII	angiotensin II
AP	area postrema
ARC	arcuate nucleus of the hypothalamus
BNST	bed nucleus of the stria terminalis
BW	body weight
CCK	cholecystokinin
CNS	central nervous system
cNTS	caudal nucleus of the solitary tract
cVLM	caudal ventrolateral medulla
DBH	dopamine-beta-hydroxylase
diEN	diluted Ensure
DMH	dorsomedial nucleus of the hypothalamus
DMV	dorsal motor nucleus of the vagus
DVC	dorsal vagal complex
EN	Ensure
Ex9	Exendin-3 (9-39)
GI	gastrointestinal
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GPR10	G-protein coupled receptor 10
h	hour(s); *refers to a period of time*

hr	hour; *refers to a time of day*
i.c.v.	introcerebroventricular
IMI	intermeal interval
LD	liquid diet
LiCl	lithium chloride
MRF	medullary reticular formation
NA	noradrenaline/noradrenergic
NAcc	nucleus accumbens
NF	non-fed
PB	phosphate buffer
PF	paraformaldehyde
PrRP	prolactin-releasing peptide
PVN	paraventricular nucleus of the hypothalamus
RES-LD	restricted liquid diet
SAL	saline
SON	supraoptic nucleus
TH	tyrosine hydroxylase
VTA	ventral tegmental area

1.0 INTRODUCTION

1.1 INHIBITION OF FOOD INTAKE: SATIATION VS. SATIETY

Although energy needs are continuous, energy intake in omnivores is intermittent. In situations where food is freely available, ingestive events occur in discrete bouts referred to as meals. Therefore, cumulative food intake in a given period of time is equivalent to the product of meal number and meal size. Changes in food intake must be the result of stimulatory or inhibitory factors producing changes in meal size and/or changes in the interval between them (meal frequency) (Davis, 1989). While copious studies have been dedicated to understanding the mechanisms and signals that stimulate food intake (e.g., hunger, reward value, circadian rhythms), the widespread existence of states of chronic nutritional excess (i.e., obesity) underscores the importance of understanding signals that inhibit feeding. The research herein is one such example.

In most human societies, the pattern of ingestive bouts in a given individual is heavily influenced by environmental (e.g., culture, social) and idiosyncratic (habits, lifestyle, health-related, and genetic) factors. While understanding ingestive behavior in complex human societies is important, animal studies offer an ideal avenue to study the fundamental behaviors and biological mechanisms governing feeding behavior. It is from animal studies that the field of ingestive behavior derived its widely accepted terms "satiation" and "satiety" that refer to

the inhibition of meal size and meal frequency, respectively (Blundell and Bellisle, 2013). More specifically, satiation refers to the accumulation of inhibitory signals within a single meal (ultimately terminating it). Satiety refers to the point at which a meal ends and the continued inhibition of eating that occurs until the initiation of the subsequent meal (defining the duration of the inter-meal interval).

Satiation and satiety are not independent processes. Both are influenced by a common set of internal (e.g., sensory, cognitive, metabolic) and environmental (e.g., social, cultural) stimuli and they directly affect each other (e.g., the size of a meal certainly influences the amount of time until the next one). However, in the most basic sense, satiation and satiety are successive processes governed by largely non-overlapping biological mechanisms (Figure l). Satiation is a sensory-driven process that involves the integration of mechanical, chemical, and hormonal signals that are generated when ingesta contacts the GI tract. On the other hand, satiety involves the integration of intestinally-derived signals with information about adiposity, energy expenditure, metabolic status, circadian rhythms, etc, that begins at the point of meal termination (Davis, 1989; Smith, 1996; Blundell and Bellisle, 2013). Therefore, keeping this distinction in mind offers a useful approach for studying the effect of experimental manipulations on feeding behavior and for better understanding the basic neural mechanisms that underlie meal size versus meal number.

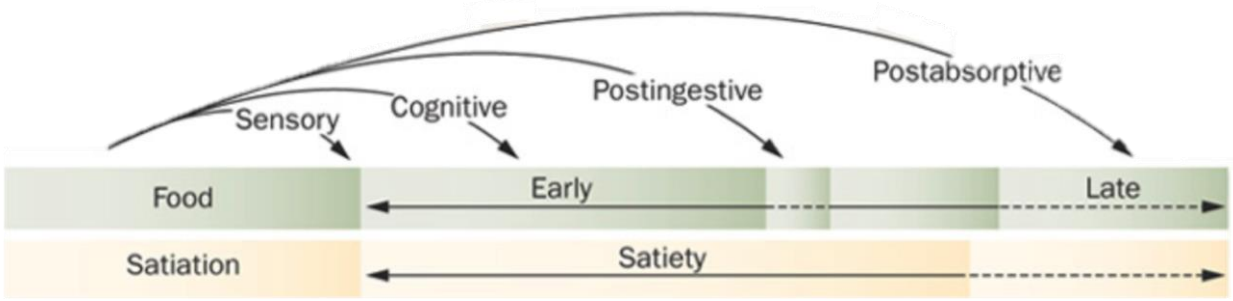

Figure 1. Feeding-related feedback signals: satiation vs. satiety

A series of multimodal negative feedback signals promote the inhibition of food intake during and after a meal. The successive and distinct nature of these signals supports the conceptualization of satiation and satiety (Blundell, 2010).

1.2 BIOLOGICAL BASIS OF SATIATION: PIONEER STUDIES

Meal number is regulated by forebrain and hypothalamic circuits that encode information about environmental events and context, learned associations, reward value, and energy status between meals. Conversely, meal size regulation is a sensory-driven process that arises from direct contact between ingested food and sensory receptors along the GI tract (Smith, 1996; Smith, 1998; Woods and Ramsay, 2000; Morton et al., 2006). Early landmark studies in rats undergoing sham-feeding were influential in revealing the peripheral origin of these feeding-generated sensory signals and their specific effects on feeding behavior.

During sham-feeding procedures, a surgically placed gastric cannula actively removes ingested food as it enters the stomach, allowing experimenters to dissociate the contributions of orosensory/pregastric (those arising from the mouth, tongue, throat, esophagus, etc.) versus gastric/postgastric (those arising from the stomach and intestines) sensory signals on feeding behavior. These studies consistently demonstrated that rats undergoing their first experience with sham-feeding displayed similar intake and licking behavior during the initial minutes of sham-fed meals as did rats feeding normally. However, these sham-fed meals were substantially larger than regular meals (Davis and Campbell, 1973; Young et al., 1974; Kraly et al., 1978). Together these observations indicated that post-gastric nutrient contact provides an important unlearned inhibitory signal that determines meal size, while orosensory (pre-gastric) signals are sufficient to initiate and maintain feeding.

Similar localization studies have been applied to the central nervous in order to better understand the neurological mechanisms that promote satiation. Pioneer work in the chronic decerebrate (CD) rat performed in the 1970-1990s implicated the hindbrain as home to the central networks that govern satiation (Grill and Norgren, 1978a; Seeley et al., 1994; Smith,

2000). Decerebration refers to the complete surgical isolation of the hindbrain from the forebrain via a knife cut through the rostral brainstem (just rostral to the cerebellum), abolishing the reciprocal communication between forebrain and hindbrain structures (Figure 2).

Despite possessing the locomotor capabilities and oral motor control involved in procuring and consuming food, CD rats do not begin eating unless food is placed directly in their mouths, indicating that the brainstem alone is insufficient for meal initiation. However, CD rats do clearly stop eating (consume distinct meals) (Grill and Norgren, 1978a; Seeley et al., 1994; Smith, 2000) and adjust the size of those meals based on palatability of the food (Grill and Norgren, 1978b; Grill and Kaplan, 1992). Together, these observations indicate that the brainstem only responds to sensory signals that arise upon nutrient contact with the GI tract (i.e., is not sufficient to support the motivational drive to procure food). However, the brainstem is sufficient to integrate positive and negative feedback signals upon feeding commencement and inhibit the motor patterns that drive feeding. Conversely, the CD rat is unable to perform more complex controls of eating such as adjusting feeding behavior based on learned associations, food availability, or metabolic status, (Grill and Norgren, 1978a; Seeley et al., 1994; Grill and Kaplan, 2002) indicating that descending input from the forebrain is required for these adjustments. Together, studies in CD rats provided critical evidence that the hindbrain is sufficient to respond to the sensory signals generated during feeding (and thus perform satiation) but requires descending input from other brain structures when adjusting food intake to internal or external stimuli [reviewed in (Seeley et al., 1994; Smith, 2000, Woods 1990; Grill and Kaplan, 2002), also see Figure 19].

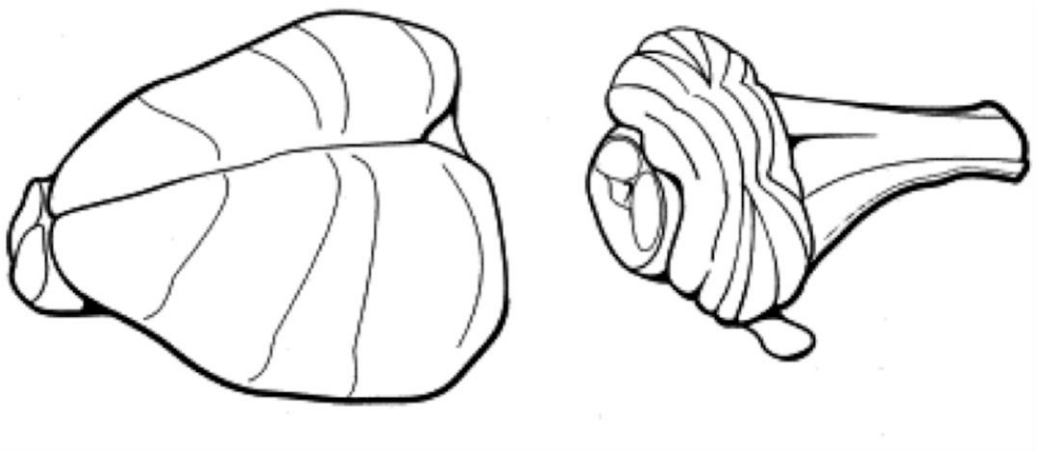

Figure 2. Schematic depicting decerebration

Space between the rat forebrain (left) and hindbrain (right) emphasizes the dissociation between the structures produced by decerebration. Behavioral studies in decerebrate rats revealed that the hindbrain contains all of the necessary neural equipment to integrate feeding-generated sensory signals and subsequently terminate a meal. Figure from (Smith, 2000).

1.3 NEURONAL PHENOTYPES OF THE CAUDAL NUCLEUS OF THE SOLITARY TRACT (CNTS): GLP-1 AND PRRP SECOND SECTION

Pioneer studies implicated the brainstem as a critical structure in the regulation of meal size. However, the neuronal phenotypes that participate in hindbrain and forebrain circuits that control feeding behavior (including satiation) are just beginning to be unraveled. Within the caudal brainstem, neurons that reside in the cNTS are the first to receive and process ingestion-related visceral sensory signals (Shapiro and Miselis, 1985; Altschuler et al., 1989b; Rinaman et al., 1989; Appleyard et al., 2007; Grill and Hayes, 2009, 2012). Although the cNTS is neurochemically diverse, the present work focuses on two neuronal populations that we propose play a role in satiation: one that synthesizes glucagon-like peptide-1 (GLP-1) and another comprises a subset of noradrenergic (NA) A2 neurons that also express prolactin-releasing peptide (PrRP). A2 (including PrRP) and GLP-1 cell groups give rise to the most widely-distributed projections to medullary, mesencephalic, diencephalic, and limbic regions of all cNTS neuronal cell groups considered thus far [reviewed in (Rinaman, 2010, 2011; Grill and Hayes, 2012), see also Section 2.3]. Therefore, A2 and GLP-1 neurons represent a route through which interoceptive feedback from the body can influence feeding behavior. A role for these neuronal populations in physiological food intake is unclear.

1.4 ANATOMICAL DISTRIBUTION OF GLP-1 AND PRRP-POSITIVE NEURONS

Prolactin-releasing peptide was identified as an endogenous ligand for the human orphan G-protein-coupled receptor hGR3/GPR10 (from here on referred to as GPR10), and earned its name

because it induces prolactin secretion from anterior pituitary cells *in vitro* (Hinuma et al., 1998). However, PrRP is absent from the external layer of the median eminence (Maruyama et al., 1999; Morales et al., 2000), and there is no evidence that endogenous PrRP plays any physiological role in prolactin release. Instead, PrRP is produced by a (large) subset of A2 neurons in the cNTS and has demonstrated roles in stress and energy regulation (see Section 2.4).

Neurons that express GLP-1 and PrRP are phenotypically distinct, but co-distributed within the cNTS extending from the upper cervical spinal cord through the caudal area postrema (AP, Figure 3A). A2 neurons can be visualized using immunohistochemical labeling of dopamine-beta hydroxylase (DBH), the rate-limiting enzyme of norepinephrine synthesis. Therefore, the PrRP-positive subset of A2 neurons can be visualized using co-immunolocalization of DBH and PrRP (Chapter 3, Appendix A). Within the cNTS, PrRP-positive neurons cluster in medial and commissural subnuclei of the cNTS, whereas GLP-1 neurons are primarily localized in the lateral subnucleus (Figure 3 B,C). Besides those in the cNTS, PrRP-positive neurons comprise a subset of the A1 NA cell group within the caudal ventrolateral medulla (cVLM), and a small group of PrRP-positive neurons are present in the dorsomedial hypothalamus (DMH) (Chen et al., 1999; Iijima et al., 1999; Maruyama et al., 1999). GLP-1 neurons are also scattered in regions somewhat dorsal and medial to the A1 cell group in the medullary reticular formation (MRF) (Larsen et al., 1997; Vrang et al., 2007; Vrang and Grove, 2011).

Many reports cited in the present Introduction leave open the possibility that functions ascribed to central signaling by PrRP-positive and/or GLP-1 neurons include signaling from neurons located not within the cNTS, but within the MRF/cVLM and/or the PrRP-expressing

neurons in the DMH. However, limited evidence suggests that GLP-1 and PrRP neurons within the cNTS vs. those in other brainstem/hypothalamic regions project to different brain areas or maintain separate functions, although this possibility should be examined. For example, NA and GLP-1 neurons within the cNTS receive direct visceral sensory input (Appleyard et al., 2007; Hisadome et al., 2011), whereas those in the MRF do not. In the absence of specific evidence to discriminate between PrRP or GLP-1 neurons within the cNTS vs. those in adjacent brainstem regions, a conservative approach dictates that projections and functions ascribed to chemically distinct neurons in either region should be considered likely to be shared by neurons in the other region. It also remains unclear whether subsets of cNTS PrRP and GLP-1 neurons project to different targets or the degree to which PrRP and GLP-1 neurons collateralize.

The cNTS is the "visceral" NTS, which is distinct from the more rostral "gustatory" NTS (Figure 3A). The cNTS is a component of the dorsal vagal complex (DVC), which also includes the AP and dorsal motor nucleus of the vagus (DMV, Figure 3B). The DVC is critical for autonomic and endocrine functions, because it relays visceral, hormonal, and somatic information from the body to the brain, thereby regulating stress responses, and energy balance. The AP is a circumventricular organ that is permeable to a wide variety of circulating factors. It contains intrinsic neurons as well as afferent projections to the cNTS that serve to translate chemical and hormonal signals from the periphery into neural signals. Sensory input from the respiratory, cardiovascular and gastrointestinal viscera also arrive via glutamatergic vagal sensory afferents. These vagal afferents synapse on cNTS GLP-1 and NA neurons (presumably at least some of which also express PrRP (Bailey et al., 2006; Appleyard et al., 2007; Hisadome et al., 2010, 2011). The cNTS also receives relayed synaptic input from the viscera via other brainstem nuclei (trigeminal, reticular) (Menétrey, 1987; Altschuler et al., 1989b; Menétrey,

1990) although whether these inputs are onto GLP-1 and PrRP neurons specifically is unknown. Due to the proximity and intercommunication with the AP in addition to direct vagal afferent innervation, GLP-1 and PrRP neurons are well poised to integrate multimodal information arising from the viscera, including, and most relevant to this work, signals controlling food intake.

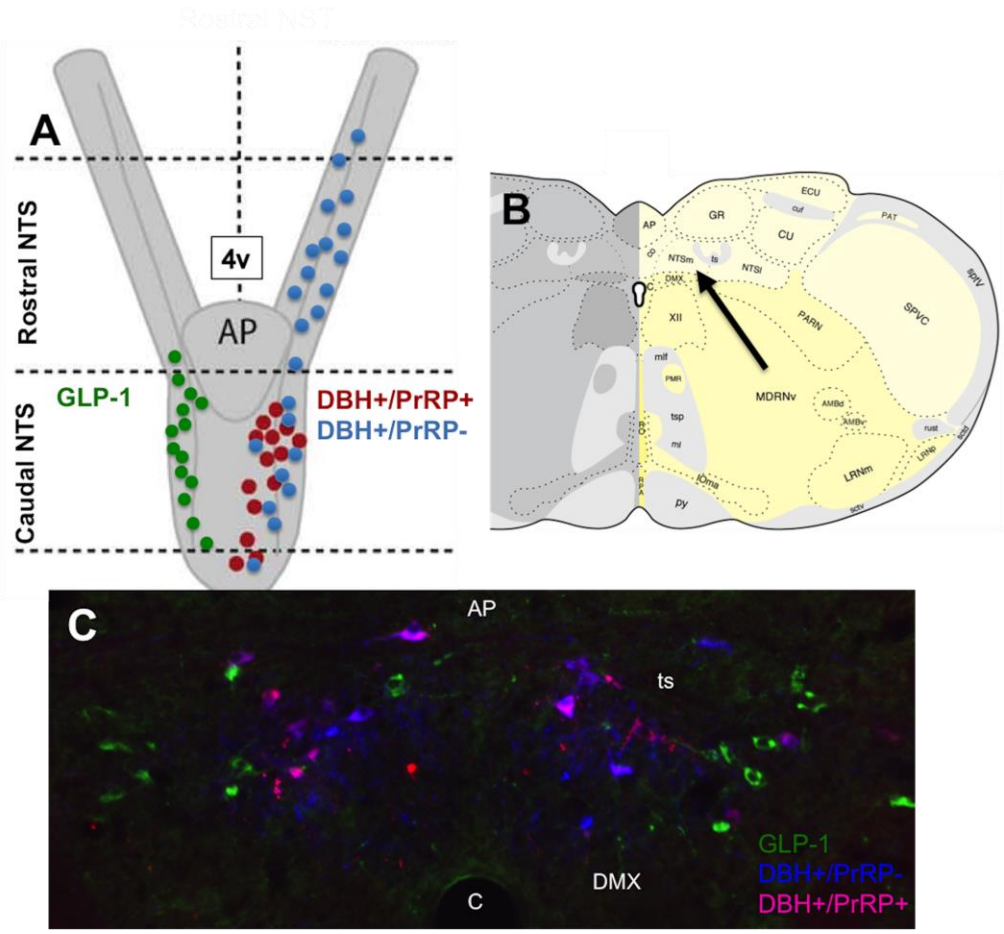

Figure 3. Anatomy of GLP-1 and DBH-positive (including PrRP-positive) neurons within the cNTS

A) Schematic depicting a dorsal view of the NTS with the approximate boundaries of the caudal ("visceral") and rostral ("gustatory") components delineated by dotted lines. Dots indicate approximate distribution of GLP-1, PrRP-positive and PrRP- negative A2 neurons. C) GLP-1-positive (green) and DBH (including PrRP)-positive neurons co-mingle within the cNTS (region indicated by black arrow in panel B) Schematics in A and B adapted from (Vrang and Larsen, 2010) and level 70 of (Swanson, 2004), respectively. *4V= 4th ventricle, AP = area postrema,*

NTSm = medial subnucleus of NTS, NTSl = lateral subnucleus of NTS, co = commissural subnucleus of NTS, DMX = dorsal motor nucleus of vagus; c = central canal, ts = solitary tract.

1.5 STIMULI THAT ACTIVATE GLP-1 AND PRRP NEURONS

Numerous studies have shown that GLP-1 and A2/PrRP neurons are activated by of experimental treatments that activate GI vagal sensory afferents and/or reduce food intake including high doses of cholecystokinin octapeptide (CCK) (Rinaman, 1999a; Maniscalco and Rinaman, 2013), LiCl (Rinaman, 1999a; Lawrence et al., 2002), endotoxin (Rinaman, 1999a; Mera et al., 2006), hemorrhage (Morales and Sawchenko, 2003), and, of particular relevance to experiments conducted in Chapter 4, a ventricular injection of saline vehicle [(Maniscalco, in press) see also Section 5.4]. In these cited studies and many others, including studies conducted in Chapters 3-4, stimulus-induced "activation" of NA, PrRP, and/or GLP-1 neurons is generally characterized by double immunolabeling to localize nuclear cFos, the protein product of the immediate-early response gene, *cfos*, together with cytoplasmic proteins identifying the chemical phenotype of activated neurons. The studies conducted in this thesis used a binary index of cFos labeling, i.e., present as determined by visible nuclear immunolabeling in a given neuron, or absent when no nuclear immunolabeling was evident. No electronic tools were used to quantify the presence or intensity of cFos immunolabeling, although quantification was standardized by having the same researcher perform all cFos analyses.

It is important to consider that the threshold of input required to activate cFos expression may vary between neurons. Therefore, as a binary index of neural activation, the visible presence or absence of cFos immunolabeling in a given neuron poorly reflects the absolute magnitude of synaptic inputs, and provides no information regarding an individual neuron's spike frequency. In order to better understand the firing properties of GLP-1 and PrRP neurons relative to a stimulus, more detailed electrophysiological experiments are required. However, quantitative and regional analyses of neural cFos expression permit assessment of stimulus induced activation

across multiple populations of phenotypically identified neurons under control and experimental conditions, making it an ideal approach for testing hypotheses regarding neural sensitivity and/or function *in vivo*.

As demonstrated above, the collective results of studies utilizing cFos indicate that GLP-1 and PrRP-positive neurons are consistently activated by stimuli that reduce food intake. While these studies provide convincing evidence that GLP-1 and PrRP-positive neurons are sensitive to the sensory signals that accompany experimentally-administered visceral stressors (and may contribute the reduction in food intake that accompanies them) (Thiele et al., 1998; Rinaman, 1999b; Kinzig et al., 2002; Rinaman, 2003; Grill et al., 2004; Maniscalco et al., 2012b)), they do not provide insight into the sensitivities of these neurons to the visceral sensory signals generated during normal food intake. A limited number of studies have reported higher proportions of activation among phenotypically-identified GLP-1 or DBH (including PrRP)-positive neurons in rats that have consumed a meal compared to compared to rats that did not eat (Rinaman et al., 1998; Takayanagi et al., 2008; Gaykema et al., 2009). However, only one of these studies (Rinaman et al., 1998) has examined the potential relationship between activation of DBH-positive neurons and the amount of food consumed, and that study did not examine the PrRP-positive subset specifically. The relationship between amount consumed and neuronal sensitivity is particularly important given the proposed role for GLP-1 and DBH (including PrRP)-positive neurons in transducing feeding-related sensory signals to the brain during satiation, which consists of an on-going accumulation of inhibitory sensory signals (Section 1.1).

1.6 EFFERENT TARGETS OF GLP-1 AND PRRP NEURONS

Prolactin-releasing peptide- and GLP-1-positive neurons are well positioned to participate in vago-vagal reflexes that modulate gastrointestinal motility, pancreatic hormone release, and other digestive-related autonomic processes associated with ingestion. Indeed, anatomical studies have shown that A2 (Hermann et al., 2005; Travagli et al., 2006; Pearson et al., 2007), including PrRP (Iijima et al., 1999) and GLP-1-positive (Llewellyn-Smith et al., 2011; Llewellyn-Smith et al., 2013) neurons project locally within the DVC, MRF, and spinal cord, regions that contain pre-autonomic neurons implicated in the control of autonomic function related to digestion. Functional studies have supported a role for PrRP (Grabauskas et al., 2004) and GLP-1 (Gulpinar et al., 2000) signaling within the brainstem in the regulation of gastric and intestinal motility. PrRP (Iijima et al., 1999) and GLP-1 positive (Larsen et al., 1997; Rinaman, 2010) axon varicosities are also present in spinal cord, pontine and medullary reticular nuclei that contain the pattern generators, pre-motor and motor neurons that control the somatic motor function associated with feeding (e.g., licking, chewing, swallowing, (Travers and Rinaman, 2002). Phenotypically-unidentified neurons within the PrRP/GLP-1-rich regions of cNTS and MRF have been shown to be synaptically linked to motor neurons that control feeding-relevant oral movements (Travers et al., 1997; Travers et al., 2010). It is unknown whether this group of neurons includes PrRP and/or GLP-1-positive neurons. It is also unknown whether or not PrRP and GLP-1 neurons (directly) communicate with each other. Nonetheless, PrRP and GLP-1-positive neurons are ideally positioned to participate in the ingestive motor circuits that shape satiation.

Dual-labeling retrograde tracing and fiber lesion studies indicate that A2 (including PrRP-positive) and GLP-1 neurons also project to multiple higher brain regions implicated in

behavioral and physiological components of food intake (Rinaman, 1999a; Morales et al., 2000; Sarkar et al., 2003; Renner et al., 2010; Rinaman, 2010, 2011; Renner et al., 2012). Immunohistochemical and *in situ* hybridization analyses reveal particularly dense GLP-1 and PrRP terminal fields and/or receptor mRNA expression in the paraventricular hypothalamus (PVN), DMH, arcuate hypothalamus (ARC), supraoptic nucleus (SON), dorsomedial thalamus, and bed nucleus of the stria terminalis (BNST) (Dodd and Luckman, 2013) (Shughrue, 1996; Merchenthaler et al., 1999; Rinaman, 2010). However, results from studies using CD rats (Section 1.2) suggest that these PrRP/GLP-1 fibers are not necessary for meal termination. This does not preclude an important role for such projections in the modulation of basic sensory integration and motor output that comprise satiation (Figure 19).

1.7 PHARMACOLOGICAL AND GENETIC MANIPULATION OF CENTRAL GLP-1 AND PRRP SIGNALING AND THEIR EFFECTS ON FOOD INTAKE

Due to its very short half-life (~ 2 minutes), peripherally-released GLP-1 is unlikely to bind to central GLP-1 receptors. Furthermore, PrRP is produced in relatively small amounts in the periphery (mostly in the intestines and adrenal gland) compared to in the caudal brainstem (Fujii et al., 1999). Therefore, the effects of receptor manipulation described in this section are most likely attributable to blocking GLP-1 and PrRP signaling of brainstem (and/or hypothalamic for PrRP) origin. However, the absence of a GLP-1 radio ligand has prohibited formal analyses of peripherally administered GLP-1 binding efficiency in the CNS. The ability of peripherally-released PrRP to impact CNS receptor function has not been investigated.

Studies using pharmacological stimulation of GLP-1 receptor and GPR10 consistently report reductions in food intake in rats and mice (Tang-Christensen et al., 1996; Turton et al., 1996; McMahon and Wellman, 1997; Asarian et al., 1998; McMahon and Wellman, 1998; Lawrence et al., 2002; Schick et al., 2003a; Abbott et al., 2005; Hayes et al., 2008; Williams et al., 2009; Onaka et al., 2010; Hayes et al., 2011a; Williams et al., 2011; Dickson et al., 2012; Kanoski et al., 2012). While important in revealing a potential role for these receptors in feeding, results of studies using this approach are not good indicators of a physiological role for these neuropeptide systems in food intake. To better understand the role of endogenous GLP-1 signaling in satiation and long-term energy balance, one research group used a knockdown strategy in which short hairpin RNA was microinjected into the cNTS of adult rats to suppress endogenous GLP-1 expression; this produced a significant and long-lasting increase in daily food intake and body weight compared to control rats (Barrera et al., 2011). However, it might be argued that the brainstem surgery itself in that study had a marked and long-lasting effect to reduce food intake and body weight gain, and that knockdown of GLP-1 expression merely attenuated the deleterious effects of surgery (see Section 5.3 and Appendix B regarding satiation vs. stress-induced hypophagia). Evidence challenging a physiological role for GLP-1 signaling in daily food intake control comes from one study using GLP-1R−/− mice, which are lean and consume a similar number of daily calories compared to wild-type mice. However, GLP- 1R−/− mice display an apparent disturbance in satiation, such that termination of food intake is delayed early in the dark period, thereby prolonging the initial nocturnal meal. This is followed by a later suppression of intake to achieve caloric compensation (see Figure 5A in Scrocchi et al., 2000).

GPR10-deficient mice display late onset hyperphagia and obesity under *ad lib* feeding conditions (Gu et al., 2004; Mochiduki et al., 2010), but not in a one-time 16 h fasting/re-

feeding protocol (Gu et al., 2004). In addition, GPR10 is required for the ability of exogenously administered PrRP and CCK to inhibit food intake in mice (Bechtold and Luckman, 2006). In another study, PrRP-deficient mice displayed increased meal size (but not frequency) under *ad lib* feeding conditions, increased intake after deprivation, and reduced responsiveness to the feeding-suppressive effects of exogenous CCK and leptin (Takayanagi et al., 2008), which endogenously function as satiety signals.

Genetic knockouts provide valuable information for a role of endogenous GLP-1/PrRP signaling in long term energy balance. However, because satiation and long-term changes in energy metabolism are modulated by similar factors (see Section 1.1), it can be difficult to separate a contribution of the manipulated signaling system to long-term energy balance vs. satiation in experiments using genetic knockouts. Furthermore, such models are susceptible to developmental and compensatory effects that can make results difficult to interpret. Lastly, these approaches have not yet been applied to rats.

Pharmacological administration of GLP-1 receptor antagonist Exendin-3 (9-39) (Ex9) offers a simpler mechanism by which endogenous signaling can be acutely suppressed. Parenchymal administration of Ex-9 into subregions of the mesolimbic reward system, i.e., the ventral tegmental area (VTA) or nucleus accumbens (NAcc), increases short-term intake of chow, palatable high-fat diet, and sucrose in *ad lib*- fed rats (Dossat et al., 2011; Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2013), suggesting that GLP-1 signaling in these regions may normally act to suppress reward-driven intake. Ex-9 targeted to the lateral hypothalamus enhances short term food intake in *ad lib*-fed rats (Schick et al., 2003a). Conversely, Ex-9 injections targeted to the fourth ventricle or cNTS increase the amount of food consumed by rats after gastric distension, but not after intestinal nutrient infusions (Hayes et al., 2009). Results

from these studies support the view that endogenous GLP-1 signaling suppresses or limits food intake across a variety of experimental conditions, and the cNTS/hindbrain may be an especially sensitive site of action for this effect (Grill and Hayes, 2012). Interestingly, several studies have reported that central Ex9 administration fails to further increase food intake in food-deprived rats (Turton et al., 1996; Schick et al., 2003a; Williams et al., 2009). Upon considering novel results of studies conducted in this thesis, I speculate as to the reason for this discrepancy in the General Discussion (Section 5.4). Of the studies that have reported increased cumulative intake after central Ex9 administration, only one has provided convincing evidence that such treatment affects meal size, specifically (Dossat 2013). However, this study did not report whether the Ex9-induced increase in meal size resulted in increased cumulative intake. There is currently no pharmacological tool with which to antagonize GPR10. Therefore, the effects of acute disruption of endogenous PrRP signaling have been poorly investigated.

A potential interpretational problem in the studies cited above is the typical comparison of data from surgically manipulated and/or drug-infused rats with data from control rats subjected to sham surgery and/or infused with vehicle. While these are appropriate experimental controls, they are incomplete. The manipulations employed in these studies are often complex, requiring one or more surgical sessions (e.g., to equip animals with chronic brain cannulas or intravenous catheters), and acute handling for central or systemic drug injection. Such manipulations are themselves likely to promote some degree of stress-induced hypophagia, such that "baseline" food intake measured in animals after central or systemic vehicle treatment may be less than intake that would be observed under non-manipulated conditions. Accordingly, recent studies in our lab have shown that the mild stress associated with injection procedures activates GLP-1 neurons above the level of injected controls (Maniscalco, in press). Therefore,

the ability of centrally administered Ex-9 or anti-PrRP antibody to increase food intake relative to vehicle-injected controls could be interpreted as evidence that central GLP-1 or PrRP signaling promotes stress-induced hypophagia (Appendix A) rather than normal food intake.

GLP-1 and PrRP-positive neurons are sensitive to variety of stimuli and blocking their receptor activity attenuates hypophagic responses to a variety of stressors (Appendix B). Therefore, it is particularly important to include non-manipulated controls in order to distinguish a contribution of GLP-1/PrRP signaling to normo-physiological food intake vs. a role in counter-acting the hypophagic effects of experimental treatments. Furthermore, more work is necessary to better understand whether endogenous central GLP-1/PrRP signaling plays a role in meal size, meal number, or both.

1.8 DO GLP-1 AND PRRP NEURONS PARTICIPATE IN SATIATION?

Satiation is a brainstem-mediated process comprised of the integration of feeding-generated sensory cues from the GI tract with input regarding environmental, metabolic, cognitive, and conditioned information from the forebrain and digestive organs. A growing body of literature demonstrates that GLP-1 and PrRP-positive A2 neurons that reside in the cNTS are sensitive to multi-modal visceral sensory signals and may participate in central networks that control food intake. However, such studies have not provided convincing evidence for a role for GLP-1 and PrRP signaling in day-to-day feeding, nor if that role is to limit meal size and/or frequency.

Therefore, we propose that GLP-1 and PrRP neurons are sensitive to the visceral sensory signals generated during feeding and thus are a route through which information about feeding status is conveyed to the CNS. Furthermore, we hypothesize that GLP-1 signaling subsequently

contributes to the termination of a meal. My dissertation work, comprising the studies that follow, tested two critical components of this central hypothesis.

In Chapter 3, I hypothesized that GLP-1 in cNTS and A2 (including PrRP-positive) neurons would be activated in rats undergoing their first experience with a 24h fast followed by access to a palatable liquid meal. Indeed, results indicated that both populations of neurons were sensitive to food intake and such activation was tightly correlated with the volume consumed. However, the relationship between neuronal activation and volume consumed was different for PrRP-positive A2, PrRP-negative A2, and GLP-1 neurons, suggesting differential sensitivities to feeding-generated signals across these three populations. However, in rats that consumed the largest meals (5 %BW), only 45% of PrRP neurons and 30% of GLP-1 neurons were activated.

Therefore, in Chapter 4, we trained rats to consume even larger (> 5 %BW) meals in order to challenge the hypothesis that such meals would further activate GLP-1 and PrRP neurons. Furthermore, we used pharmacological blockade of GLP-1 receptors in *ad lib*-fed rats to challenge a causal role for GLP-1 signaling in satiation under these feeding conditions. In that study, data from non-injected rats was compared to data in rats injected with Ex9 and vehicle in order to reveal a role for endogenous GLP-1 signaling in satiation rather than in attenuating decreases in food intake associated with vehicle injection. Results obtained in Chapter 4 were consistent with our hypothesis: larger meals (12-13 %BW) produced more robust activation (up to 90%) of GLP-1 and PrRP neurons. Blocking GLP-1 signaling increased food intake compared to baseline intake, which was driven by a preferential increase in meal size.

Together, results from my dissertation work demonstrate than GLP-1 and PrRP neurons effectively track the amount consumed. Also, in addition to reversing the hypophagia associated with vehicle injection, GLP-1 signaling contributes to satiation, and not satiety, by increasing

meal size and duration. However the effect to increase meal size occurred only once a substantial amount of food had already been consumed, supporting a role for endogenous GLP-1 signaling in promoting satiation selectively when food intake becomes exceptionally large. It remains unclear, however, which GLP-1 receptor populations are mediating such effects and whether/how endogenous PrRP signaling also contributes to satiation (see Section 6.0).

2.0 DIFFERENTIAL ACTIVATION OF GLP-1 AND PRRP-EXPRESSING NA NEURONS IN THE CAUDAL NUCLEUS OF THE SOLITARY TRACT IN NON-ENTRAINED RATS AFTER INTAKE OF SATIATING VS. NON-SATIATING MEALS

2.1 INTRODUCTION

Food intake is the product of meal size and meal number (Smith, 1996). Meal number is regulated by cortical, limbic, striatal, and hypothalamic forebrain circuits, and is modulated by signals that convey information about environmental events, learned associations, reward, and energy status between meals [see (Woods and Seeley, 2000; Morton et al., 2006) and Section 1.1 for reviews]. Conversely, meal size is controlled primarily at the level of the caudal brainstem (Smith, 1996), and is modulated by satiety signals arising from the GI tract and related digestive organs during the ingestion and digestion of food; these signals are conveyed largely by vagal sensory inputs to the NTS (Grill and Norgren, 1978a; Grill and Smith, 1988; Appleyard et al., 2007; Hayes et al., 2009; Grill, 2010; Hisadome et al., 2011; Grill and Hayes, 2012). The NTS relays feeding-related visceral sensory signals to the forebrain, as well as to brainstem pattern generators and pre-motor neurons that control the motoric components of feeding (i.e., licking, chewing, and swallowing (Smith, 2000; Travers and Rinaman, 2002). Thus, NTS neurons are critically involved in receiving and processing GI satiety signals that terminate ingestive consummatory behaviors, thereby limiting meal size (Rinaman, 2010).

NTS neurons that receive and process satiety signals are neurochemically diverse (Rinaman, 2010). However, recent studies have implicated two intermingled but phenotypically distinct caudal NTS neuronal populations in meal size control: noradrenergic (NA) neurons that comprise the A2 cell group, and neurons that are immunopositive for glucagon-like peptide-1 (GLP-1) (Rinaman, 2010; Vrang and Larsen, 2010; Rinaman, 2011). Based on increased expression of the immediate-early gene product cFos, results in laboratory rats indicate that A2 and GLP-1 neurons are stimulated by experimental treatments that activate GI vagal sensory afferents with synaptic inputs to the caudal NTS (Rinaman et al., 1998; Rinaman, 1999a; Vrang et al., 2003; Gaykema et al., 2009; Maniscalco and Rinaman, 2013). Such treatments include mechanical gastric distension (Vrang et al., 2003) and systemic administration of CCK (Rinaman et al., 1993; Rinaman et al., 1995; Rinaman, 1999a; Maniscalco and Rinaman, 2013). A2 neurons also are activated in a meal size-dependent manner by voluntary food intake in rats that have been acclimated/entrained to a feeding schedule in which repeating cycles of overnight food deprivation are followed by a predictable morning re-feeding period (Rinaman et al., 1998). Conversely, the same repeating schedule of food deprivation followed by a large anticipated meal does not activate GLP-1 neurons (Rinaman, 1999a), although GLP-1 neurons are activated in rats after a variety of interoceptive stressors (Rinaman, 1999a). Feeding schedule entrainment is associated with anticipatory physiological adjustments that serve to limit the homeostatic challenge of consuming large meals, thereby reducing the interoceptive stress that would otherwise be produced by the meal, and permitting increased meal size (Woods, 1991; Woods and Ramsay, 2000). Since multiple lines of evidence support the view that central GLP-1 signaling suppresses food intake (Asarian et al., 1998; Hayes et al., 2009; Barrera et al., 2011; Zhao et al., 2012), the lack of GLP-1 neuronal recruitment in meal-entrained rats that consume a

large anticipated meal may reflect homeostatic adjustments that minimize interoceptive stress. Indeed, attenuated feeding-induced activation of GLP-1 neurons may contribute to the progressively larger meals consumed by rats during meal entrainment (Woods, 1991; Woods and Ramsay, 2000). In non-entrained rats, such anticipatory physiological adjustments are absent, and deprivation-induced food intake is more directly limited by GI distension and other sensory feedback generated by the acute homeostatic challenge of consuming a large unanticipated meal.

The present study was designed to test the hypothesis that both A2 *and* GLP-1 neuronal populations are recruited in non-entrained rats after voluntarily intake of a large, unanticipated meal. To challenge this hypothesis, we examined cFos activation among DBH- and GLP-1-positive caudal NTS neurons in rats after deprivation-induced intake of unrestricted or restricted volumes of a palatable liquid diet (i.e., Ensure). We extended our analysis of feeding-activated neurons to include a specific caudal subset of DBH-positive NTS neurons that co-express prolactin-releasing peptide (PrRP) along with NA synthetic enzymes (Chen et al., 1999; Iijima et al., 1999). Central PrRP signaling is implicated in stress responses and control of energy balance (Matsumoto et al., 2000; Maruyama et al., 2001; Samson et al., 2003; Gu et al., 2004; Ohiwa et al., 2007; Toth et al., 2008; Mochiduki et al., 2010; Onaka et al., 2010), and PrRP neurons may participate in meal size regulation (Lawrence et al., 2000; Lawrence et al., 2002; Bechtold and Luckman, 2006). PrRP-positive neurons within the caudal NTS are activated in experimentally naïve rats and mice after overnight food deprivation followed by re-feeding (Takayanagi et al., 2008), although that report did not examine the potential relationship between the amount of food consumed and the extent of PrRP neuronal recruitment.

2.2 MATERIAL AND METHODS

2.2.1 Animals and feeding protocol

Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (Harlan; n=31; 200-270g BW) were individually housed in hanging wire cages in a temperature-controlled room with lights on from 07:00-19:00 hr. Food (Purina rat chow #5001) and water were available *ad lib*, except as noted for each experiment. Rats were acclimated to these conditions for at least 48h before experiments were initiated. The test diet was liquid Ensure (Creamy Milk Chocolate or Homemade Vanilla; both 1.06 kcal/g; 14% protein, 64% carbohydrate, 22% fat by kcal; Abbott Nutrition, Columbus, OH). A pilot study revealed no flavor-related differences in 30-min intake by rats (n=12) after overnight food deprivation, consistent with a previous report of similar *ad lib* intake of chocolate vs. vanilla Ensure by male Sprague-Dawley rats (Archer et al., 2006). All rats in the present study were pre-exposed overnight to a ball-tipped drinking spout attached to a graduated cylinder containing 10 ml of Ensure (in addition to chow and water) in order to reduce neophobic responses to the drinking spout and test diet during subsequent deprivation-induced feeding. Every rat consumed all of the available Ensure during this pre-exposure. In some cases, up to 5 ml of the 10 ml provided for overnight pre-exposure was inaccessible, either because the drinking spout leaked (as evidenced by a small volume of dried Ensure found on the pan beneath the wire cage floor the next morning), and/or because the inner metal tube extended a bit higher than the volume remaining at the bottom of the cylinder. Thus, pre-exposure volumes varied somewhat across rats, but did not vary systematically across experimental feeding groups.

Several days after Ensure pre-exposure, chow was removed from cages between 15:00 and 16:00 hr; water was not removed. Twenty-four hours later, food-deprived rats were weighed and then given 30 min home-cage access to Ensure (the same flavor to which they had been pre-exposed) in one of three feeding conditions, or served as food-deprived/non-fed controls, as follows:

1. Unrestricted access to Ensure (i.e., rats were given access to a volume in excess of any rat's 30 min intake)
2. 30% restricted (i.e., rats were given 70% of the average volume consumed in 30 min by rats in the "unrestricted access" group)
3. 50% restricted (i.e., rats were given 50% of the average volume consumed in 30 min by rats in the "unrestricted access" group)
4. Food deprived/non-fed controls (i.e., no Ensure)

Our pilot study confirmed that most food-deprived rats with unrestricted access to Ensure (n=12) voluntary terminated intake within the first 15 min, whereas some rats consumed a few more mls during the subsequent 15 min period (direct observation). However, at the end of 30 min, none were still actively consuming Ensure. Thus, rats with 30 min unrestricted access to Ensure were able to feed to satiety (i.e., as evidenced by voluntary termination of intake), whereas rats in the two restricted groups were limited to consuming smaller defined volumes during the same 30 min period. At the end of the 30 min feeding period, the amount of Ensure consumed by each rat was recorded and feeding bottles were removed.

2.2.2 Perfusion/tissue preparation

Rats were sacrificed one hour after the end of the 30 min feeding period. This time point was selected to capture peak cFos activation in response to ingestion and satiation, since cFos protein immunolabeling peaks 60-90 min after neural stimulation and persists at peak levels for at least 30 additional min (Chaudhuri et al., 2000). Rats were deeply anesthetized with pentobarbital sodium (*Fatal Plus*; 100 mg/kg BW, i.p., Butler Schein, Columbus, OH) and then transcardially perfused with 50-100 mL saline followed by 100 mL of 2% paraformaldehyde (PF; Sigma, St. Louis, MO) containing 1.5-2.0% acrolein (Polysciences Inc., Warrington, PA), followed by 100 mL of 2% PF. After perfusion, clamps were placed at the distal esophagus and proximal duodenum, stomachs were excised, and gastric contents removed and weighed. Brains were post-fixed overnight *in situ* in 2% PF at 4°C, then removed from the skull, blocked, cryoprotected in 20% sucrose, frozen and sectioned at 35 μm using a sliding microtome. Sections were collected serially in six sets that each contained a complete rostrocaudal series of sections spaced by 210 μm. Sections were stored at -20°C in cryoproservant solution (Watson et al., 1986) to await immunohistochemical processing.

2.2.3 Immunohistochemistry

Tissue sections were removed from cryopreservant, rinsed in 0.1 M phosphate buffer (PB, pH 7.2), pre-treated in 0.5% sodium borohydride (Sigma) solution for 20 min, rinsed in PB, immersed in 0.5% H_20_2 for 10 min, and rinsed again in PB. Primary and secondary antisera were

diluted in PB containing 0.3% Triton X (Sigma), 1% donkey serum (Jackson ImmunoResearch, West Grove, PA), and (when noted) 1% bovine serum albumin (BSA; Sigma).

For dual cFos and GLP-1 immunolabeling, one set of pre-treated sections from each rat was incubated overnight in rabbit anti-GLP-1 at room temperature, using one of two primary antisera that yielded similar GLP-1 neural labeling (Peninsula Laboratories, San Carlos, CA, #T-4057, 1:8000 with BSA, or Peninsula #T-4363, 1:10000, no BSA). After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 1 hr at room temperature, rinsed, then incubated in avidin-biotin complex (Vectastain Elite reagents, Vector Labs, Burlingame, CA) for 1.5 hrs. After rinsing, tissue underwent an H_20_2-catalyzed reaction in a solution of diaminobenzidine (DAB; Sigma) in 0.1M Tris buffer to produce a brown cytoplasmic GLP-1 peroxidase reaction product. GLP-1-labeled tissue was then incubated overnight at room temperature or for 48-72 hr at 4^{o}C in one of two primary rabbit antisera raised against cFos protein: one was provided by Drs. Philip Larsen and Jens Mikkelsen, Panum Institute, Denmark (1:5000), and the second was purchased from EMD Chemicals (PC38, San Diego, CA; 1:2000). Pilot studies confirmed that both cFos antisera produced similar results, including similar within-animal cFos activation counts for GLP-1 neurons. To visualize nuclear cFos labeling, sections were rinsed, incubated overnight in CY3-conjugated donkey anti-rabbit IgG (1:300, Jackson Immunochemicals), and rinsed again to produce an immunofluorescent signal.

For triple immunolabeling of cFos, dopamine beta hydroxylase [DBH; to identify all A2 and C2 neurons within the caudal NTS; (Rinaman, 2011)], and PrRP, an adjacent set of pre-treated tissue sections from each rat was incubated in rabbit anti-cFos (1:20,000; EMD Chemicals) followed by donkey anti-rabbit IgG and avidin-biotin reagents, as described above.

After rinsing, tissue underwent an H_20_2-catalyzed reaction in a solution of 0.1M sodium acetate buffer containing DAB and 2.5% nickel sulfate (Sigma) to produce a blue/black nuclear cFos peroxidase reaction product. Reacted sections were then rinsed and incubated in a cocktail of rabbit anti-PrRP (#H-008-52, 1:1000, Phoenix, Burlingame, CA) and mouse anti-DBH (#MAB308, 1:5000, Millipore, Billerica, MA) primary antisera overnight at room temperature. After rinsing, sections were incubated overnight at 4ºC in a cocktail of CY3-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated donkey anti-mouse IgG (both at 1:300, Jackson ImmunoResearch).

After immunocytochemical processing, labeled tissue sections were rinsed in PB and mounted onto adhesion Superfrost Plus Microscope Slides (Brain Research Laboratories, Waltham, MA), allowed to dry, then dehydrated and defatted in a series of graded ethanols followed by xylene. Slides were coverslipped with Cytoseal 60 mounting medium (Fisher Scientific, Pittsburgh, PA) and stored at room temperature in covered boxes.

2.2.4 Quantification of feeding-induced neural activation

3.2.4a. GLP-1 neural activation: GLP-1 immunoperoxidase and cFos immunofluorescent labeling was visualized using an Olympus BX51 microscope equipped with brightfield and epifluorescence (X-Cite 120) optics. In each rat, all GLP-1 neurons within the caudal NTS were photographed in sections (spaced by 210 μm) from the upper cervical spinal cord through the mid-level of the area postrema using a 20X objective and a Hamamatsu camera. GLP-1 neurons within the MRF were not included in cell counts. Photographs of GLP-1 neurons were uploaded into Adobe Photoshop, and were identified visually on a computer screen based on brown

cytoplasmic peroxidase labeling that was clearly perikaryal, rather than dendritic or axonal. By overlaying the brightfield channel (containing visible GLP-1 neurons) and the fluorescent channel, GLP-1 neurons were further classified as cFos-positive if their nucleus contained visible fluorescent cFos immunolabeling, regardless of intensity (see Figure 4). GLP-1 neuronal activation was quantified as the proportion (percent) of GLP-1-positive NTS neurons that also were cFos-positive.

3.2.4b. Activation of DBH+PrRP-positive (PrRP-positive) and DBH+PrRP-negative (PrRP-negative) neurons: DBH and PrRP dual immunofluorescence and cFos immunoperoxidase labeling was visualized using epifluorescence and brightfield optics. In photographs from each animal, DBH-positive neurons were identified and counted within the caudal NTS from the upper cervical spinal cord through sections just rostral to the area postrema, where the NTS remains immediately adjacent to the wall of the 4th ventricle. DBH-positive neurons were further classified as PrRP-positive or PrRP-negative based on the presence or absence of dual DBH+PrRP immunofluorescent labeling, and were identified as cFos-positive when their nucleus contained visible blue-black immunoperoxidase labeling, regardless of intensity (see Figure 4). As previously reported (Chen et al., 1999), all PrRP-positive NTS neurons also were DBH-positive, whereas many DBH-positive NTS neurons were PrRP-negative, especially in sections through and rostral to the rostral area postrema.

2.2.5 Data Analysis

Analyzed data included the amount of Ensure consumed by each rat (expressed as volume, and

also as weight, with the latter converted to %BW), the postmortem weight of gastric contents, the percentage of the consumed amount and the total volume of Ensure that emptied from the stomach before gastric contents were collected post-perfusion, and the proportions of identified neurons (i.e., GLP-1, PrRP-positive, and PrRP-negative) activated to express cFos. Data are presented in graphs and tables as group mean ± SE. Statistically significant effects of feeding group on experimental outcomes were identified using ANOVA, with feeding group as the independent variable, followed by post-hoc LSD T-tests. Differences were considered statistically significant when $p < 0.05$. Correlational analyses also were performed to examine relationships between Ensure intake and cFos activation of GLP-1 and/or PrRP-positive or -negative neurons.

2.3 RESULTS

The number of rats per feeding condition group is indicated in Table 1. ANOVA confirmed no significant between-group differences in post-deprivation, pre-meal BWs (Table 1).

Table 1: Meal-related statistics in non-entrained rats on the final test day

Bodyweights, meal-related data, and gastric content data for each of the four feeding condition groups. Values represent group mean ± SE. Different superscript letters (i.e., a, b, c) indicate significant difference between groups within a column as determined by ANOVA and post-hoc T-tests ($p < .05$).

Feeding condition	Pre-meal BW (g)	Meal size (mL)	Meal size (%BW)	Caloric value of meal (kcal)	Post-mortem weight of gastric contents (g)	% of meal retained in stomach at time of perfusion	Amount of Ensure emptied from stomach by time of perfusion (mL)
Unrestricted (n=10)	230.10 ± 6.49[a]	9.00 ± .84[a]	4.74 ± .50[a]	9.54 ± .89[a]	6.47 ± .79[a]	66.98 ± 3.43[a]	2.98 ± .20[a]
30%restricted (n=6)	217.67 ± 4.40[a]	7.00 ± .13[b]	3.15 ± .06[b]	7.42 ± .14[b]	4.38 ± .24[b]	62.04 ± 4.74[a]	2.62 ± .34[a]
50%restricted (n=8)	232.13 ± 4.58[a]	4.28 ± .48[c]	2.13 ± .15[b]	4.50 ± .49[c]	1.34 ± .27[c]	30.63 ± 4.00[b]	2.95 ± .37[a]
Non-fed (n=7)	216.86 ± 5.89[a]	0.00[d]	0.00[c]	0.00[d]	0.00[c]	-	-

2.3.1 Meal sizes and post-mortem gastric contents

Except for pre-meal BW, all meal-related statistics listed in Table 1 showed an overall effect of feeding condition as determined by one-way ANOVA ($p < .001$ for all meal-related statistics). Rats within the unrestricted feeding group consumed approximately 4.7% of their BW within 30 min, whereas rats in the 30% and 50% restricted feeding groups consumed approximately 3.2% and 2.1% of their BW, respectively (Table 1). These intake amounts corresponded to approximately 9.5 kcal in the unrestricted group, 7.4 kcal in the 30% restricted group, and 4.5 kcal in the 50% restricted group (Table 1). Rats did not consume water during the Ensure re-feeding period. There were no flavor-related (i.e., chocolate vs. vanilla) differences in intake within any group. All rats in the unrestricted group had excess available but unconsumed Ensure remaining after the end of the 30 min feeding period, whereas all rats in the two restricted feeding groups consumed the entire volume of available Ensure. The post-mortem weight of gastric contents (collected 1h after the end of the 30-min feeding period) ranged from approximately 1.3 to 6.5 g, Table 1). Only ~31% of Ensure consumed by rats in the 50% restricted feeding group remained within the stomach post-perfusion, whereas ~62% of the consumed amount remained in the 30% restricted feeding group, and ~67% remained in the unrestricted feeding group. However, the absolute volume of Ensure that emptied from the stomach was similar across feeding groups (~2.6-3.0 ml, Table 1), as expected based on previous research (Kalogeris et al., 1983). Thus, post-gastric nutrient signals were similar across feeding groups.

2.3.2 Feeding-induced neuronal activation

Relatively few GLP-1 or DBH+ neurons were cFos-positive in unfed control rats, whereas cFos labeling increased within both neuronal populations in rats that were allowed to feed (Figs. 5 and 6). ANOVA revealed a significant main effect of feeding condition on the proportion of GLP-1-positive neurons expressing cFos [$F(3, 27) = 12.02$, $p < 0.001$], the proportion of PrRP-positive neurons expressing cFos [$F(3, 25) = 40.70$, $p < 0.001$], and the proportion of PrRP-negative neurons expressing cFos [$F(3, 25) = 23.54$, $p < 0.001$].

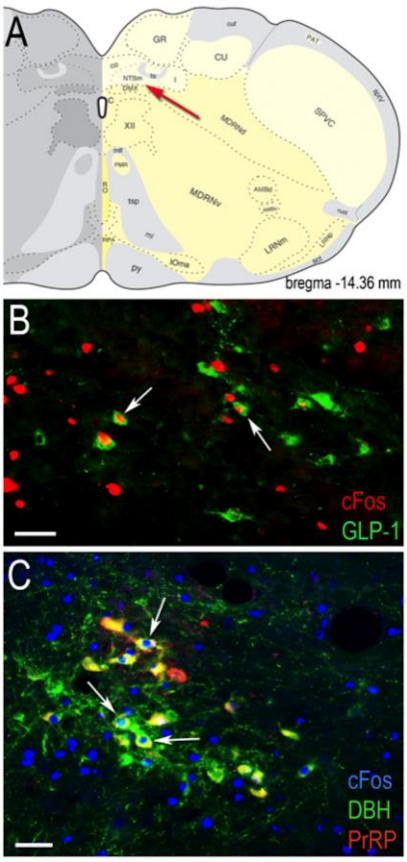

Figure 4. Examples of GLP-1+cFos and DBH+PrRP+cFos immunolabeling in cNTS

GLP-1-positive (B) and PrRP-positive (C) neurons within the cNTS (region indicated by red arrow in panel A) are activated to express cFos in representative rats that consumed an unrestricted (B) or 30% restricted (C) Ensure meal after food deprivation. White arrows point out some of the double-labeled neurons visible within each photomicrograph, in which nuclear cFos immunolabeling is co-localized with cytoplasmic GLP-1 or DBH+PrRP immunolabeling. Brain schematic image in panel A adapted from (Swanson, 2004). Scale bars in B and C = 50 μm.

2.3.3 GLP-1 neuronal activation

The proportion of GLP-1-positive neurons expressing cFos within each feeding condition group is presented in Figure 5. There was no main effect of feeding condition on the number of identified GLP-1-positive neurons per section ([$F(3, 27) = 1.52$, $p = 0.23$). Very few GLP-1 neurons were cFos-positive in non-fed control rats (n=7), or in rats that were allowed to consume either 50% restricted (n=8) or 30% restricted volumes of Ensure (n=6; Figure 5). Conversely, approximately 28% of GLP-1 neurons were c-Fos-positive in rats that consumed unrestricted volumes of Ensure (n=10; Figure 5). Post-hoc analyses revealed no between-group differences in GLP-1 cFos activation among non-fed controls (1.95% ± 0.75), 30% restricted (0.90% ± 0.52), and 50% restricted (2.00% ± 0.58%) groups. The proportion of GLP-1 neurons expressing cFos in rats consuming unrestricted volumes of Ensure (27.57% ± 6.11) was significantly greater than in each of the other three groups ($p < 0.001$ for each comparison). Thus, intake of only a satiating, unrestricted volume of Ensure was sufficient to activate GLP-1 neurons above levels quantified in non-fed control rats, even though rats in the 50% and 30% restricted feeding condition groups consumed 2-3% of their BW during the 30-min feeding period, and even though similar volumes of Ensure had emptied from the stomach into the small intestine in rats from all three feeding groups (Table 1).

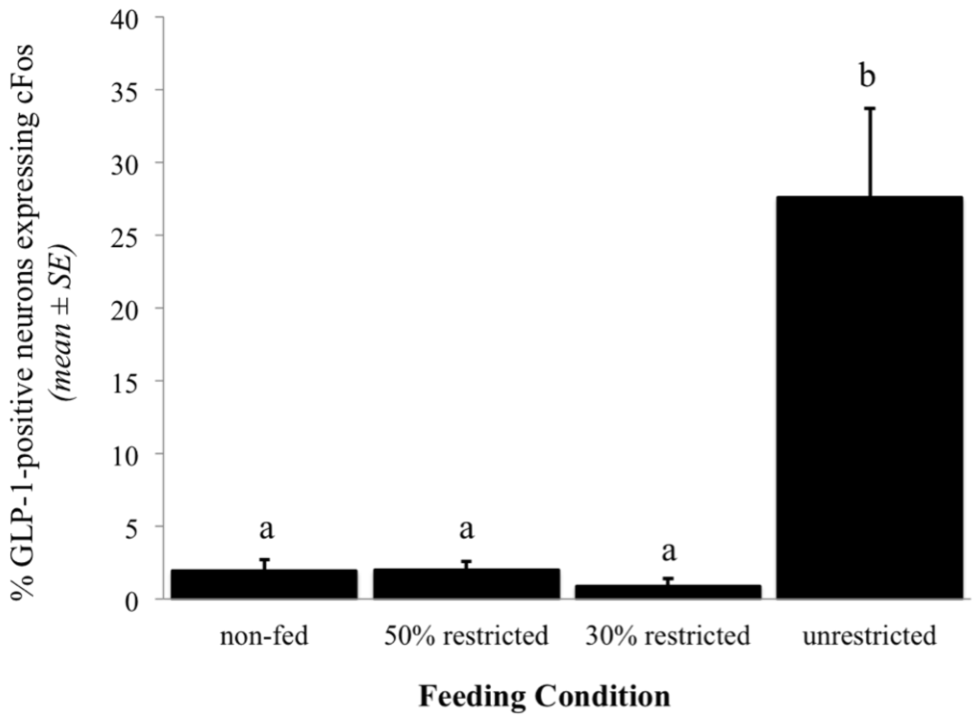

Figure 5. GLP-1 neuronal activation in meal-entrained rats

Percentage of GLP-1-immunopositive cNTS neurons that express cFos immunoreactivity in response to meals of increasing size (see Table 1). Different letters indicate statistically significant differences in cFos activation between feeding condition groups ($p < .05$).

2.3.4 Activation of PrRP-positive and PrRP-negative neurons

Tissue sections were damaged in two rats (one from the 50% restricted group, one from the unrestricted group) that were included in GLP-1 activation analyses, and thus these two rats did not contribute data for analysis of DBH-positive neurons. There was no main effect of feeding condition on the number of identified DBH-positive neurons per section that were PrRP-positive ([$F(3, 24) = 0.72$, $p = 0.55$) or PrRP-negative ([$F(3, 24) = 2.73$, $p = 0.07$). In non-fed control rats (n=7), DBH-positive neurons expressed very little cFos regardless of PrRP phenotype (0.37% ± 0.28 of PrRP-positive neurons and 1.36% ± 0.23 of PrRP-negative neurons; Figure 6). Similarly low activation of DBH-positive neurons was observed in the 50% restricted intake group (2.36% ± 1.70 of PrRP-positive and 3.29% ± 1.19 of PrRP-negative neurons; n=7). However, unlike the negligible GLP-1 activation observed in the 30% restricted feeding group, both phenotypic subpopulations of DBH-positive neurons displayed significantly increased cFos expression in rats consuming a 30% restricted volume of Ensure (n=6; 18.48% ± 6.06 of PrRP-positive neurons, and 10.06% ± 1.27 of PrRP-negative neurons; $p < 0.01$ compared to activation of each DBH population in 50% restricted or non-fed control rats; Figure 6). Feeding-induced activation of PrRP-positive neurons was more markedly increased in the unrestricted feeding condition group (n=9; 43.92% ± 10.68; Figure 6), and was significantly greater in the unrestricted feeding group compared to each other feeding group ($p < 0.01$ for each comparison). Among PrRP-negative neurons, cFos activation in unrestricted rats (13.24% ± 1.42) was similar to activation of the same phenotypic neural population in rats consuming a 30% restricted meal (Figure 6).

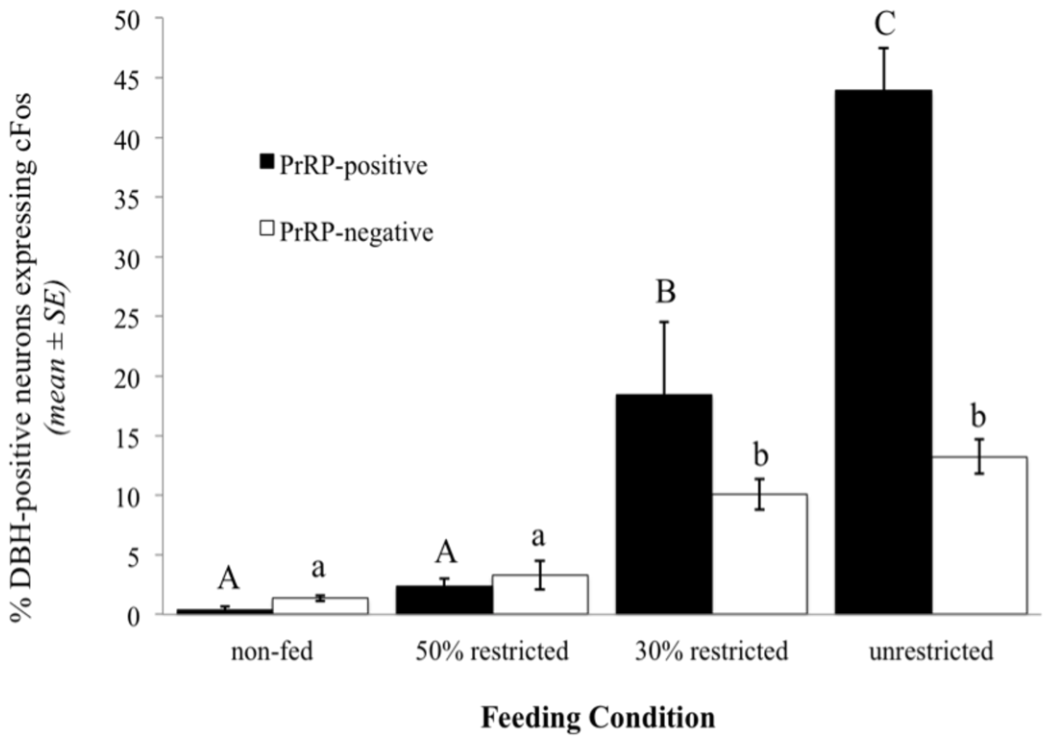

Figure 6. A2 neuronal activation in non-entrained rats

Percentage of DBH-immunopositive neurons, either PrRP-positive or PrRP-negative, that express cFos immunoreactivity in response to meals of increasing size (see Table 1). Within each phenotypic subgroup of DBH-positive neurons, different letters indicate significant differences in cFos activation between feeding condition groups ($p < .05$).

2.3.5 Correlational analyses

The amount of Ensure consumed by individual rats (expressed as % BW) across all three feeding condition groups (i.e., excluding non-fed controls) was positively and significantly correlated with the proportion of GLP-1 neurons expressing cFos (Pearson's R = 0.83; $p < 0.001$), with the proportion of PrRP-positive neurons expressing cFos (Pearson's R = 0.81; $p < 0.001$), and with the proportion of PrRP-negative neurons expressing cFos (Pearson's R = 0.61, $p = 0.002$). In addition, the proportion of GLP-1-positive neurons that expressed cFos was significantly correlated with the proportion of PrRP-positive neurons that expressed cFos across all three feeding conditions (Pearson's R = 0.71; $p < 0.001$). Since the Ensure volumes consumed by rats in the 50% and 30% restricted feeding condition groups were artificially limited to a predetermined amount, additional correlations between Ensure volumes consumed and cFos activation were performed separately for rats within the unrestricted group alone, whose intake volumes were larger but also more variable within the group (Figures 6 and 7). Once again, strong positive correlations were observed between the amount of Ensure consumed by each rat and the corresponding activation of GLP-1 neurons (Figure 7; Pearson's R = 0.74; $p < .001$) and PrRP-positive neurons (Figure 8; Pearson's R = 0.75; $p = 0.02$). The correlation between amount of Ensure consumed and activation of PrRP-negative neurons was not significant (Pearson's R = 0.12; $p = 0.76$), nor was the correlation between activation of PrRP-positive neurons and activation of GLP-1 neurons within the unrestricted feeding group alone (Pearson's R = 0.58; $p = 0.10$).

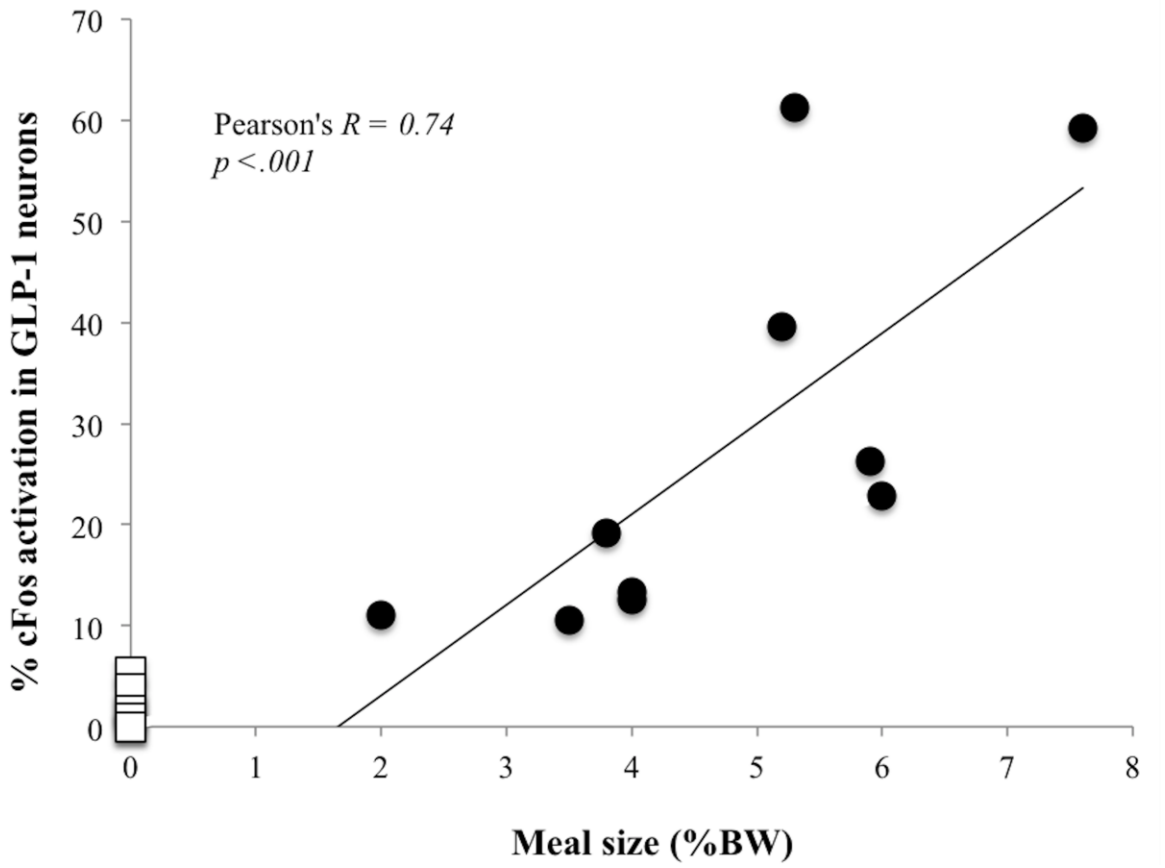

Figure 7. Meal size and activation of GLP-1-positive neurons in non-entrained rats

Relationship between meal size and percentage of GLP-1-positive neurons activated to express cFos in rats that consumed unrestricted, satiating volumes of Ensure (black dots). Data points representing non-fed controls (n=7) are added for comparison (open squares). Each symbol represents one animal. Best-fit line and correlation statistics refer only to rats in the unrestricted feeding group.

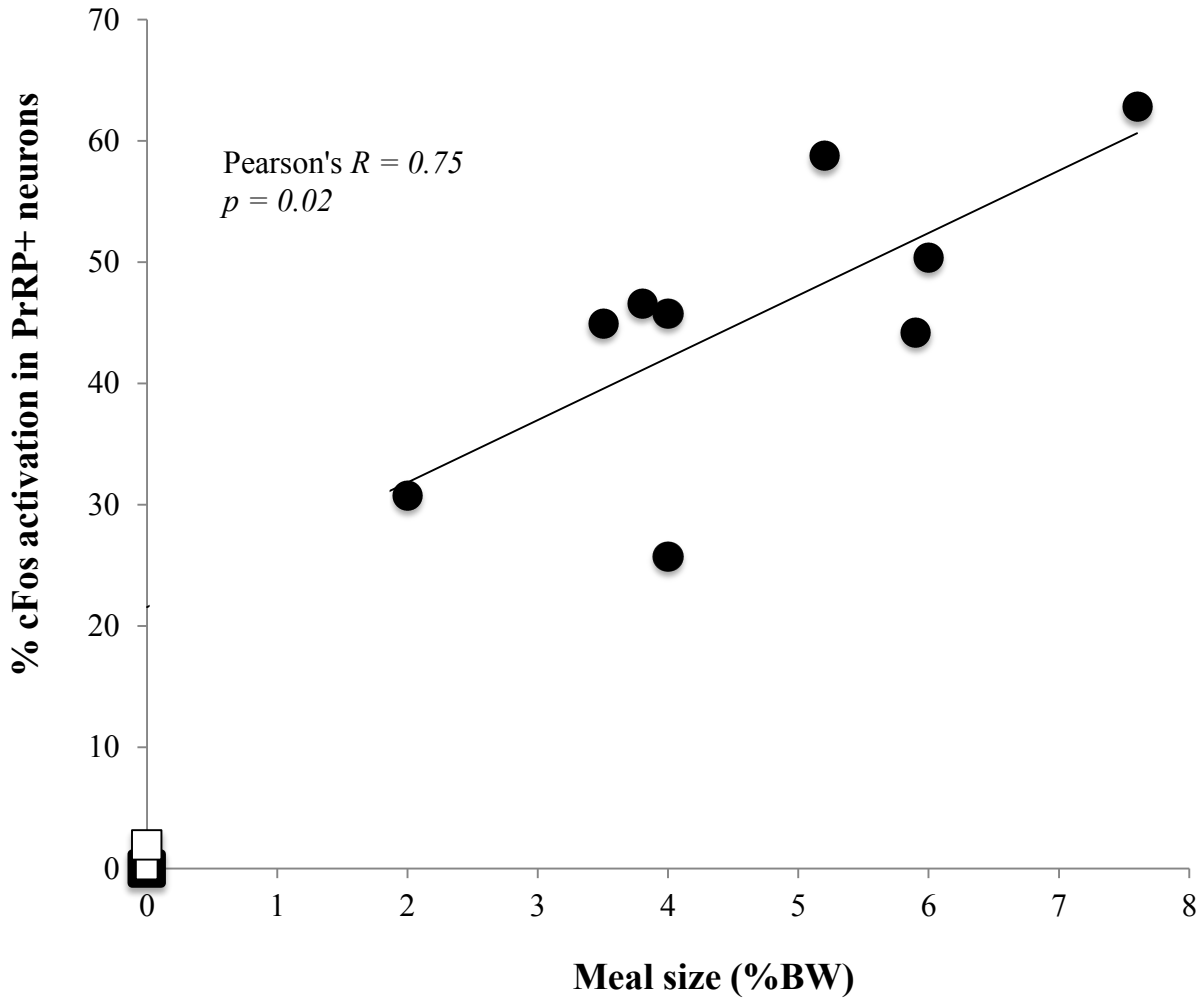

Figure 8. Meal size and activation of PrRP-positive neurons in non-entrained rats

Relationship between meal size and percentage of PrRP-positive neurons expressing cFos in rats that consumed unrestricted, satiating volumes of Ensure (black dots). Data points representing non-fed controls (n=7) are added for comparison (open squares). Each symbol represents one animal. Best-fit line and correlation statistics refer only to rats in the unrestricted feeding group.

2.4 DISCUSSION

Results from the present study demonstrate that DBH-positive neurons within the caudal visceral NTS (including primarily the A2 cell group, but also the caudal portion of the C2 cell group) are progressively recruited to express cFos in rats that consume progressively larger volumes of palatable liquid Ensure after first-time food deprivation. Among DBH-positive neurons examined in our study, the PrRP-positive subset was most sensitive to feeding-induced activation, although smaller proportions of PrRP-negative neurons also were recruited when rats consumed large volumes of Ensure. The only previous report on feeding-induced activation of PrRP-positive NTS neurons (Takayanagi et al., 2008) did not examine the relationship between amount consumed and neuronal recruitment. In the present study, food-deprived rats had to consume at least 3.1% of their BW within a 30 min period in order to significantly increase cFos expression by DBH-positive cNTS neurons. Notably, however, significant feeding-induced activation of GLP-1 neurons occurred only in the unrestricted feeding group, in which rats consumed very large volumes of Ensure that corresponded to nearly 5% of their BW. Maximal activation of the PrRP-positive subset of DBH neurons also was achieved in the unrestricted feeding group. We interpret these results as evidence that progressive recruitment of A2/C2 neurons within the caudal NTS, especially the most caudally-situated PrRP-positive subset of A2 neurons, effectively "tracks" the magnitude of GI signals and other meal-related sensory feedback. Conversely, only the largest meals are sufficient to activate GLP-1 neurons. Based on these and other published results (discussed further, below), we posit that GLP-1 neurons are relatively insensitive to normal, progressively increasing GI signals during food intake, but are ultimately recruited in response to interoceptive stress generated by the homeostatic challenge of consuming a very large, unanticipated meal. Our findings are consistent with several "loss-of-

function" studies that have implicated both GLP-1 and DBH/PrRP neuronal populations in meal size control (Grill and Kaplan, 1992; Bechtold and Luckman, 2006; Takayanagi et al., 2008; Hayes et al., 2009; Barrera et al., 2011). However, many cFos-positive cNTS neurons in the present study were not immunopositive for either GLP-1 or DBH/PrRP. We do not discount the potential contributions of these phenotypically unidentified neurons to meal termination.

Previous reports from our laboratory and others indicate that NA and GLP-1 neurons are activated to express cFos in rats after a variety of treatments that stimulate vagal sensory inputs to the caudal NTS (Rinaman et al., 1998; Rinaman, 1999a; Vrang et al., 2003; Gaykema et al., 2009; Maniscalco and Rinaman, 2013). In one of these studies, we examined the ability of food intake to activate tyrosine-hydroxylase (TH)-positive catecholamine neurons (which include but are not limited to NA neurons) within the caudal NTS (Rinaman et al., 1998). However, that study employed a different experimental paradigm, in which rats were acclimated for 5 days to a feeding schedule that included repeating cycles of overnight food deprivation followed by daily 1 h access to unrestricted amounts of solid or liquid diet. On day 5, rats were killed 30 min after the end of the 1 h feeding period, during which they consumed unrestricted or rationed amounts of food (Rinaman et al., 1998). Despite the different feeding paradigms utilized in that study and the present one, feeding-induced activation of TH-positive NTS neurons in the earlier study was similar to feeding-induced activation of DBH-positive neurons in the present study: progressively larger proportions of TH-positive NTS neurons expressed cFos in rats that consumed progressively larger amounts of food, with peak activation of TH neurons reaching ~30-40% in rats that consumed an unrestricted (satiating) amount of food (Rinaman et al., 1998). Conversely, in a follow-up study (Rinaman, 1999a) using tissue sections from the same meal-entrained rats, we found that GLP-1 neurons were not activated after scheduled feeding, even in

rats that consumed liquid diet volumes that were 2-3 times greater than those consumed by non-entrained rats in the present study. GLP-1 neurons were, however, activated in rats after several "interceptive stress" treatments, including i.p. injection of lithium chloride, lipopolysaccharide, or a high dose of CCK (Rinaman, 1999a).

Considered together with the present results in rats that were not entrained to consume large meals, these findings support the view that GLP-1 neurons are recruited by significant homeostatic challenges, including the challenge generated by consuming a large unanticipated meal (Shiraishi et al., 1984; Woods, 1991; Woods and Ramsay, 2000). However, in the present study, the average proportion of GLP-1 neurons expressing cFos in rats that consumed unrestricted meals after 24 hr food deprivation (~28%) is only slightly elevated above the ~20% of GLP-1 neurons that express cFos under "baseline", *ad lib* feeding conditions (Maniscalco and Rinaman, 2013). Thus, our results could be interpreted as evidence that after food deprivation, only large satiating meals of Ensure are sufficient to restore GLP-1 activation to "baseline" levels observed in rats maintained under *ad lib* feeding conditions.

We can only speculate as to what feature of the consumed meal serves as the primary stimulus for NA/PrRP and GLP-1 neural activation in the present study. Although rats in each feeding group consumed different volumes of Ensure, and thus different amounts of fat, protein, and carbohydrate calories, statistically similar volumes of consumed Ensure (~ 3 ml) had emptied from the stomachs in all three feeding groups by the time of perfusion. Therefore, differences in intestinal and post-absorptive nutrient signaling are unlikely to contribute to between-group differences in cFos activation. Most of this cFos activation was contained within the medial and commissural subdivisions of the caudal NTS, where distension-sensitive gastric and intestinal vagal afferents terminate (Zhang et al., 1995; Travagli et al., 2006). Thus, we posit

that between-group differences in DBH/PrRP and GLP-1 neuronal activation reflect different degrees of GI distension coded by vagal sensory inputs to the caudal NTS, where the majority of DBH-positive NA neurons (A2 cell group) are PrRP-positive. Since rats within each feeding group consumed different volumes of Ensure, we cannot rule out potential contributions of differential magnitude or duration of oral sensory and motor stimuli to between-group differences in cFos activation. However, our previous study (Rinaman, 1999a) demonstrated very low activation of GLP-1-positive neurons and similar activation of DbH-positive neurons in food-entrained rats that consumed liquid meals that were much larger than those in the present study, with more concurrent oral sensory and motor stimulation. Therefore, the contribution of oral sensory and motor signals to cFos expression within the caudal NTS is likely minimal in the present study.

In more rostral sections through the rostral area postrema, increasing numbers of PrRP-negative neurons likely include C2 neurons that are intermingled among A2 neurons at this level, and rostral to the area postrema, the large majority of DBH-positive neurons are C2 neurons that are PrRP-negative (Chen et al., 1999; Roland et al., 1999)]. Given the extreme gastric distension produced by intake of unrestricted Ensure meals, recruitment of visceronociceptive spinal afferents might contribute to increased activation of A2 and GLP-1 neurons within the caudal NTS. Indeed, some of the NTS cFos expression induced by inflation of a gastric balloon is attributable to spinal sensory afferents (Traub et al., 1996), and the spinosolitary tract conveys viscerosensory activation from the spinal dorsal horn to the cNTS (Menétrey, 1987, 1990).

Previous anatomical and functional reports demonstrate an important role for brainstem circuits in generating and modulating the motoric aspects of feeding (e.g., licking, chewing, swallowing) and accompanying vagal autonomic adjustments of GI motility and secretion

(Rogers et al., 2003; Hermann et al., 2005; Travagli et al., 2006; Pearson et al., 2007; Rinaman, 2011; Llewellyn-Smith et al., 2013). Decerebrate rats that lack brainstem-forebrain communication still exhibit satiation (Section 1.2) (Grill and Norgren, 1978a; Grill and Smith, 1988; Grill and Kaplan, 1992), demonstrating that isolated brainstem circuits are sufficient for meal termination. Our own unpublished data identify PrRP- and GLP-1-immunoreactive fibers in brainstem regions that control somatic motor and autonomic aspects of feeding, including the DVC, parabrachial nucleus, more rostral (taste) regions of the NTS, and the medullary and pontine reticular formation. It currently is unknown whether GLP-1 or PrRP-immunoreactive terminals are synaptically linked to brainstem neurons that control feeding behavior, but this question is ripe for experimental analysis.

Our results demonstrate that large satiating meals of palatable liquid Ensure recruit NA (especially PrRP-positive) and GLP-1-positive neurons within the caudal NTS in rats that have not been acclimated/entrained to a feeding schedule. Conversely, we previously reported that large meals activate similar proportions of NA neurons but do not activate GLP-1 neurons in meal-entrained rats (Rinaman et al., 1998; Rinaman, 1999a). Considered together, these findings support the conclusion that PrRP-positive A2 neurons are progressively recruited during feeding as a function of meal size, regardless of prior meal entrainment history, whereas GLP-1 neurons are recruited only by very large meals consumed by rats that have not been acclimated to scheduled feeding.

3.0 HINDBRAIN GLP-1 NEURONS TRACK INTAKE VOLUME AND PROMOTE SATIATION IN MEAL-ENTRAINED RATS

3.1 INTRODUCTION

Food intake is regulated by a complex, neurochemically diverse neural networks distributed within the brainstem, hypothalamus, and limbic forebrain. Food intake is the product of meal number (determined by signals that initiate feeding bouts) and meal size (determined by signals that terminate feeding bouts) (Smith, 1996). When rats in a controlled laboratory environment have unlimited access to standard chow, meal size is largely determined by feeding-generated satiety signals arising from the GI tract that are delivered by vagal sensory inputs to the cNTS (Grill and Norgren, 1978a; Grill and Smith, 1988; Appleyard et al., 2007; Grill and Hayes, 2009; Hayes et al., 2009; Grill, 2010; Hisadome et al., 2011). The cNTS relays satiety signals to a variety of central regions, including brainstem pattern generator and pre-motor circuits that control the motoric components of feeding (i.e., licking, chewing, and swallowing) (Smith, 2000; Travers and Rinaman, 2002). While it is clear that the cNTS is critically involved in receiving and processing signals that modulate ingestive consummatory behaviors, details regarding the neurochemical identity and circuit connectivity of cNTS neurons that limit meal size are just beginning to emerge.

Recent studies support the hypothesis that GLP-1-immunopositive cNTS neurons

participate in meal termination. In rats, GLP-1 neurons are activated to express the immediate-early gene product cFos in response to experimental treatments that reduce meal size, including restraint stress (Calvez et al., 2011; Maniscalco, 2014), i.p. injection of CCK (West et al., 1984; Rinaman, 1999a; Maniscalco and Rinaman, 2013), or gastric distension (Davis and Campbell, 1973; Vrang et al., 2003). Our recently published study explored whether food intake itself activated cFos expression by GLP-1 neurons (Kreisler et al., 2013). In that study rats were food deprived for 24 hours for the first time and then allowed to re-feed to satiety on palatable liquid Ensure. Although rats consumed approximately 5% of their body weight within 30 min, these large, satiating liquid meals activated cFos expression in only ~30% of GLP-1 neurons (Kreisler et al., 2013). By comparison, approximately 20% of GLP-1 neurons express cFos under baseline conditions in non-treated, *ad lib*-fed control rats (Maniscalco and Rinaman, 2013). Since the proportion of GLP-1 neurons expressing cFos was significantly and positively correlated with intake volume (Kreisler et al., 2013), we hypothesized that rats consuming even larger volumes would display proportionately higher levels of GLP-1 neural activation. To challenge this hypothesis, the first experiment in the present study examined GLP-1 neural activation in rats that were trained over 5 days to consume significantly larger volumes within a 1 h period, which activated up to 90% of GLP-1 neurons (see Section 4.4).

The progressive increase in GLP-1 neuronal activation assessed in rats after intake of progressively larger volumes suggests that GLP-1 neurons participate in the process of satiation. However, it also is possible that GLP-1 neurons are simply tracking gastric distension or some other feeding-related sensory signal, without actively participating in meal size control. Support for a role of GLP-1 neural signaling in satiation has been equivocal. Pharmacological antagonism of central GLP-1 receptors in rats by i.c.v. infusion of Ex9 has been reported to increase

cumulative intake in some feeding paradigms (Turton et al., 1996; Hayes et al., 2009; Williams et al., 2009; Dossat et al., 2011; Alhadeff et al., 2012), but not in others (Turton et al., 1996; Hayes et al., 2009; Williams et al., 2009; Alhadeff et al., 2012). For example, Ex9 administered into the 4th ventricle increased chow intake compared to intake by vehicle-injected rats, but only after rats had first consumed 9 ml of Ensure (Hayes et al., 2009). Intake by non-injected rats was not reported in that study, making it unclear whether GLP-1 receptor antagonism increased baseline intake or simply countered a hypophagic effect of handling or i.c.v. vehicle injection. Similarly, another group reported convincing evidence that i.c.v. Ex9 dose-dependently increases cumulative dark-onset food intake in *ad lib*-fed rats (Dossat et al., 2011), although comparable intake by the same rats under non-injected baseline conditions was not reported. In another set of studies, rats that were food-deprived for the first time subsequently consumed the same amount of chow regardless of whether they were pretreated i.c.v. with Ex9 or vehicle before refeeding (Turton et al., 1996; Williams et al., 2009). Of all the published studies that have examined food intake in rats after central administration of Ex9, only one has reported an effect of Ex9 to increase meal size (Dossat et al., 2013). However, that study also did not report comparable intake data from non-injected rats, and did not report whether the Ex9-induced increase in meal size resulted in increased cumulative intake.

Based on the differential ability of smaller (Chapter 3) vs. larger (present study) satiating liquid meals to activate cFos expression by GLP-1 neurons, we hypothesized that endogenous central GLP-1 receptor signaling might contribute to satiation only when food is consumed in excess of homeostatic need. To explore this, we administered Ex9 or vehicle i.c.v. to *ad lib*-fed rats that were trained to consume liquid Ensure for a limited period of time each day, and assessed Ex9 effects on subsequent meal-related parameters and cumulative intake. We also

compared baseline Ensure intake to intake in rats after vehicle or Ex9 injection in order to examine whether endogenous GLP-1 signaling attenuates hypophagic responses to the mild stress of handling and i.c.v. injection.

3.2 MATERIAL AND METHODS

Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague Dawley rats (Harlan, Indianapolis, IN) were individually housed in hanging wire cages in a temperature-controlled room (lights on from 0700–1900 h), with *ad lib* access to food (Purina rat chow #5001) and water, unless otherwise noted. Rats were acclimated to this environment for at least 3 days prior to experimental manipulations.

3.2.1 Experiment 1: Feeding-induced GLP-1 neuronal activation in rats entrained to consume a standard liquid diet (LD) for 1 h.

3.2.1a. Meal entrainment. Ad lib-fed rats (n = 29; 230-300g BW at time of final testing) were pre-exposed overnight to a ball-tipped drinking spout attached to a graduated cylinder containing 10 ml of Ensure (Milk Chocolate flavor, 1.06 kcal/g; 14% protein, 64% carbohydrate, 22% fat by kcal; Abbott Nutrition, Columbus, OH) in addition to chow and water. This pre-exposure was performed to reduce neophobic responses to Ensure during a subsequent deprivation-induced feeding test in a subset of rats, described below. Every rat consumed all of the available Ensure

during overnight pre-exposure. While only a subset of rats was assigned to an Ensure feeding group on the final test day (see below), all rats were pre-exposed similarly in order to standardize their treatment histories.

At least 48h later, all rats were fasted overnight (~22 hr, beginning at ~1600 hr) to initiate a 5-day meal-entrainment (scheduled feeding) protocol. Fasting was discontinued after this initial night. On the first and subsequent days, rats received access to an excess volume of LD (0.5 kcal/ml; 20% protein, 70% carbohydrate, 10% fat by kcal; Research Diet, New Brunswick, NJ) presented in their home cage for 1 h each afternoon (beginning at 1430 h), with the amount consumed recorded on each of the 5 acclimation days (Figure 9). Water was removed during LD access. On the first and subsequent days, one and a half to two hours after the end of LD access (17:00-17:30hr), rats were given 12-13g of chow (3.0 kcal/g) to consume overnight. Each rat consumed the entirety of its chow ration by the next morning on each of the 5 acclimation days. Rats maintained or increased their BW by up to 5g per day during the 5-day acclimation period. One hour LD intake and BWs were recorded daily.

3.2.1b. Final treatment groups. On day 6, meal-entrained (schedule-fed) rats were divided into 5 experimental groups in order to examine cFos activation in meal-entrained rats consuming test meals of different volumes and caloric densities. One group received their usual 1 h access to an unrestricted volume of LD (LD rats, n = 6). A second group received 1 h access to an unrestricted volume of Ensure, which was diluted by 50% with water to match the caloric density of LD (0.5 kcal/ml, diEN rats, n = 5). A third group received 1 h access to an unrestricted volume of *un*diluted Ensure (1.06 kcal/ml, EN rats, n = 5). A fourth group received 1 h access to a restricted volume of LD (RES-LD rats; n = 7), equivalent to 60% of the average LD volume

consumed by all rats on the previous day (i.e., on day 5 of the feeding schedule). The fifth group of rats was not fed during the final 1 h period (NF rats; $n = 6$).

3.2.1c. Perfusion fixation. Thirty to 45 minutes after the end of the final 1 h feeding period, rats were deeply anesthetized with pentobarbital sodium (Fatal Plus; 100 mg/kg BW, i.p., Butler Schein, Columbus, OH) and then transcardially perfused with 50–100 ml saline followed by 100 ml of 2% paraformaldehyde (PF, Sigma, St. Louis, MO) containing 1.5–2.0% acrolein (Polysciences Inc., Warrington, PA), followed by 100 ml of 2% PF. After perfusion, clamps were placed at the distal esophagus and proximal duodenum, stomachs were excised, and gastric contents removed and weighed. Brains were post-fixed overnight *in situ* in 2% PF at 4°C, then removed from the skull, blocked, cryoprotected in 20% sucrose, frozen and sectioned at 35 μm using a sliding microtome. Sections were collected serially in six sets such that each contained a complete rostrocaudal series of sections spaced by 210 μm. Sections were stored at −20°C in cryoproservant solution (Watson et al., 1986) to await immunohistochemical processing.

3.2.1d. Immunohistochemistry. Tissue sections were removed from cryopreservant, rinsed in 0.1 M phosphate buffer (PB, pH 7.2) and pre-treated in 0.5% sodium borohydride followed by 0.5% H_2O_2. For dual cFos and GLP-1 immunoperoxidase labeling, one set of pre-treated sections from each rat was incubated overnight at room temperature or for 48–72 h at 4 °C in primary rabbit antiserum raised against cFos protein (EMD Chemicals, San Diego, CA, #PC38; 1:20,000). After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; 1:500) for 1 h at room temperature, rinsed, then incubated in avidin–biotin complex (Vectastain Elite reagents, Vector Labs, Burlingame, CA) for 1.5 h.

Diaminobenzidine (DAB; Sigma) intensified with nickel sulfate was catalyzed by H_2O_2 to produce a blue-black nuclear cFos reaction product.

After cFos immunolabeling, sections were incubated overnight in rabbit anti-GLP-1 (Peninsula, San Carlos, CA, #T-4363; 1:10,000) at room temperature. After rinsing, sections were incubated in biotinylated donkey anti-rabbit IgG and then avidin–biotin complex, as described above. After rinsing, tissue underwent an H_20_2-catalyzed reaction in a solution of DAB in 0.1 M Tris buffer to produce a brown cytoplasmic GLP-1 peroxidase reaction product.

3.2.1e. Quantification of feeding-induced GLP-1 neuronal activation. In each rat, the number of GLP-1 neurons within the cNTS and MRF was quantified in sections (spaced by 210 μm) from the upper cervical spinal cord through the mid-level of the area postrema using a 20x objective on a light microscope. GLP-1 neurons were classified as cFos-positive if their nucleus contained visible cFos immunolabeling, regardless of intensity. GLP-1 neuronal activation was quantified as the proportion (percent) of GLP-1-positive cNTS or MRF neurons that also were cFos-positive.

3.2.1f. Data analysis. Analyzed data included the amount of LD, diEN, or EN consumed by each rat (expressed as volume, and also as weight, with the latter converted to % BW), the postmortem weight of gastric contents, the percentage of the consumed amount and the total amount of diet (expressed as kcal) that emptied from the stomach before gastric contents were collected post-perfusion, and the proportions of identified GLP-1 neurons activated to express cFos within the cNTS and the MRF. Data were combined by final feeding group on day 6 and are presented in graphs and tables as group mean ± SE. Statistically significant effects of feeding

group on experimental outcomes were identified using ANOVA, with feeding group as the independent variable, followed by planned post-hoc Tukey t-tests (corrected for multiple comparisons) to detect differences between individual feeding groups. The Tukey post-hoc test was used because it is relatively conservative, which is appropriate given the large number of comparisons performed between the 6 experimental groups. Differences were considered statistically significant when $p < 0.05$. Correlational analyses also were performed to examine relationships between feeding-related measures and cFos activation of GLP-1 neurons.

3.2.2 Experiment 2: Ensure intake after GLP-1 receptor blockade in meal-entrained rats

This experiment was designed to evaluate the role of endogenous GLP-1 signaling in controlling liquid Ensure intake in meal-entrained rats.

3.2.2a. Cannulation procedures. Experimentally naive, individually-housed male rats (n = 13, 287-308g BW at time of surgery) were anesthetized with isoflurane (1.5–2% in oxygen) and placed into a stereotaxic frame. Rats were fitted with a unilateral chronic indwelling 21-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) positioned above the lateral ventricle, 1.5 mm lateral and 0.90 mm caudal to bregma, with the tip protruding 2.7 mm below the surface of the skull. Cannulas were fixed to the skull with anchor screws and dental acrylic and fitted with removable obturators that sat flush with the tip of the guide cannula. Correct cannula placement was verified 5-6 days later, after pre-surgery bodyweights were attained. For this purpose, water-replete rats were injected i.c.v. with 2 µl of sterile 0.15M saline (SAL) containing 5 ng of angiotensin II (ANGII, Sigma). All rats included in this report began drinking

water within 30 sec and drank at least 6 ml in 30 min, evidence for accurate cannula placement (Johnson and Epstein, 1975).

3.2.2b. Meal-entrainment protocol using lickometer-equipped feeding boxes. Beginning 2-7 days after the ANGII screening test, cannulated rats (now 292-344 g BW) were acclimated to a feeding schedule that was maintained for 7 days, as follows: from 1600-1800 h each day, rats were individually placed into clear Plexiglass feeding boxes (12 x 10-inch floor, 8-inch height) with stainless steel rod floors (Med Associates, Georgia, VT) in a room adjacent to the housing room. Feeding boxes contained a contact lickometer connected to a metal ball-spout and graduated cylinder that supplied unrestricted amounts of Ensure (0.93 kcal/ml). The volume of Ensure within the graduated cylinder was recorded at 0 and again at 120 min after placing the rat in the box. Lickometers were connected to a computer with software (Med PC) set to record the cumulative number of licks made by each rat every 60 sec over the 2 h feeding period. Each rat was placed in the same feeding box equipped with the same ball-spout each day. After each 2 h feeding period, rats were immediately returned to their home cages and given an excess amount of pre-weighed chow that they could consume *ad lib* overnight. Each morning (between 0900-1000hr), remaining chow and spillage were collected and weighed to determine intake, and rat BWs were recorded. During daily collection of BW data, rats were gently restrained by hand and cannula obturators manipulated in order to acclimate rats to procedures used during subsequent i.c.v. injections.

3.2.2c. Intracerebroventricular injections and Ensure intake. On day 7 of the feeding schedule, rats were randomly assigned to receive an i.c.v. infusion of either 3 µL of saline vehicle (SAL, n

= 7) or vehicle containing 100 µg Ex9 (Ex9, n = 6), a dose previously reported to increase food intake after i.c.v. infusion in adult rats compared to intake after i.c.v. vehicle under certain experimental conditions (Turton et al., 1996; Kanoski et al., 2011). Infusates were administered at approximately 1530 hr using a Hamilton syringe attached to polyethylene tubing attached to a 26-gauge injector tip (Plastics One) that extended 2.5 mm beyond the tip of the guide cannula, to enter the ventricular space. After i.c.v. infusion rats were returned to their home cages for 15-30 min, then were placed into the lickometer chambers from approximately 1600-1800 hr for 2 h Ensure intake as usual.

After completing the experiment, to confirm proper i.c.v. cannula placement and patency, rats were deeply anesthetized with Fatal Plus, infused i.c.v. with ~5 µl of black India ink, and then transcardially perfused with 100-150 ml of paraformaldehyde (PF, Sigma, St. Louis, MO). All data included in this report were obtained from rats with visible ink at the base of the hypothalamus and within the cerebral aqueduct, confirming proper i.c.v. cannula placement and patency.

3.2.2d. Data analysis

Cumulative intake volume: The total volume of Ensure consumed by each rat (converted to grams and then expressed as % BW) during the 2 h feeding period was analyzed and compared at baseline (day 6) and after i.c.v. injection (day 7). Cumulative Ensure intake data are presented both as absolute intake values and as values normalized to each animal's baseline intake (% change from baseline). Statistically significant between-group differences in cumulative intake volume were determined using mixed ANOVA for absolute values and independent samples T-tests for normalized values.

Meal structure: Lick data were used to determine meal structure within each 2 h feeding period. Based on published criteria for rats consuming a liquid diet (West et al., 1984; Rushing et al., 1997; Aja et al., 2001), a meal was defined as at least three licks, while a pause of at least 5 min between licks defined the end of the previous meal. A previous study demonstrated that varying the pause criterion from 2 to 60 min does not significantly change the number of meals detected under such experimental conditions (Rushing et al., 1997). Analyzed data included meal number, meal size (# of licks), meal duration (minutes), and intermeal interval (IMI, minutes) during feeding under baseline (non-injected) conditions (day 6) and after either SAL or Ex9 injection (day 7). Statistically significant effects of i.c.v. injection on feeding parameters were identified using mixed ANOVA for absolute values followed by T-tests, and independent samples T-tests for normalized values.

3.3 RESULTS

3.3.1 Experiment 1: Feeding acclimation and gastric emptying of the final test meal in meal-entrained rats consuming LD

Daily 1h LD intakes for all rats during the 5-day acclimation period in Experiment 1 (as volume and %BW) are presented in Figure 9. LD intake increased progressively between days 1 and 4 (Figure 9), whereas intake on day 5 was not significantly different from intake on day 4 (paired-samples T-test: $t(28) = 1.27$, $p > 0.05$). On the final intake test and perfusion day (day 6), rats assigned to the LD group consumed approximately 12.9% of their BW (~ 31 ml; Figure 9, Table

2) within 60 min, similar to LD intake of all rats on acclimation days 4 and 5. Conversely, rats assigned to the diEN, EN, or RES-LD groups consumed smaller volumes (i.e., approximately 10.7%, 8%, and 7.5% of their BW, respectively; Figure 9). No rats were still consuming food when bottles were removed at the end of the 1 h feeding period. At the time of perfusion (30 min after the end of the 1 h feeding period), post-mortem gastric content assessment indicated that more calories had emptied from the stomach into the intestines in LD and diEN rats (~9.8 and 9.3 kcal, respectively; Table 2) compared to calories emptied in EN and RES-LD rats (~6.8 and 7.2 kcal, respectively; Table 2).

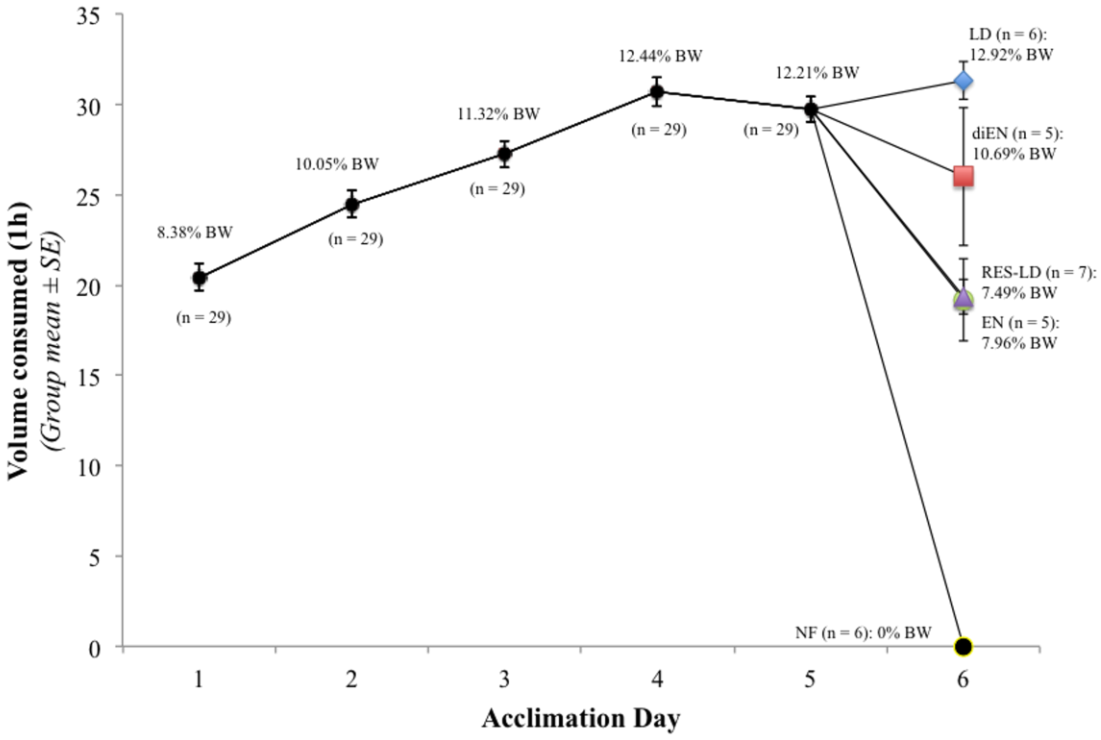

Figure 9. Average daily 1 h LD intake during the meal-entrainment acclimation period and on the final test day in Experiment 1.

Data from all 29 rats in the experiment are represented for each acclimation day (days 1-5). There was no significant difference in 1 h intake volume between days 4 and 5 (paired-samples T-test, $p > .05$). On day 6, rats were randomly assigned to one of five feeding or non-fed conditions, as indicated (colored symbols). Intake values (%BW) are indicated at each data point, and represent the average group intake on that day.

Table 2. Meal-related statistics in meal-entrained rats on the final test day (Experiment 1)

Values represent group mean ± SE. Different superscript letters indicate significant differences between groups within a column, as determined by one-way ANOVA with Tukey HSD post-hoc tests *(p* < .05*)*.

Feeding condition	Pre-meal BW (g)	Meal size (mL)	Meal size (% BW)	Post-mortem weight of gastric contents (g)	Post-mortem intestinal nutrient exposure (kcal)
Liquid diet (LD) (n = 6)	242.47 ± 2.08[a]	31.33 ± 1.05[a]	12.92 ± 0.47[a]	11.80 ± 0.38[a, b]	9.77 ± 0.63[a]
Diluted EN (diEN) (n = 5)	243.30 ± 8.76[a]	26.00 ± 3.81[a]	10.69 ± 0.67[a]	7.32 ± 1.43[a,c]	9.34 ± 0.22[a]
Ensure (EN) (n = 5)	241.40 ± 2.91[a]	19.20 ± 2.29[b]	7.96 ± 0.94[b]	12.41 ± 1.90[b]	6.80 ± 0.77[b]
Restricted LD (RES-LD) (n = 7)	258.57 ± 1.94[b]	19.36 ± 0.97[b]	7.49 ± 0.38[b]	5.01 ± 0.78[c]	7.17 ± 0.19[b]
Non-fed (NF) (n = 6)	244.00 ± 4.73[a]	0[c]	0[c]	-	-

3.3.1a. Feeding-induced activation of GLP-1 neurons in meal-entrained rats. As expected, there was no main effect of feeding condition on the number of GLP-1-positive neurons counted within either the cNTS [66.69 ± 5.08 neurons per rat; between-groups comparison $F(4, 24) = 1.82$, $p > 0.05$] or the MRF [54.41 ± 3.95 neurons per rat; between-groups comparison $F(4, 24) = 0.95$, $p > 0.05$]. Also as expected, very few GLP-1-positive neurons in either medullary region were cFos-positive in NF rats, whereas cFos expression by GLP-1 neurons in both regions was increased in rats within all four fed groups (Figure 10). GLP-1 neural activation within the cNTS in a representative rat from the LD group is shown in Figure 11. ANOVA revealed a significant main effect of feeding condition on the proportion of GLP-1 neurons expressing cFos within the cNTS [$F(4, 24) = 37.62$, $p < 0.001$] and MRF [$F(4, 24) = 11.98$, $p < 0.001$]. Within the cNTS, the proportion of GLP-1 neurons activated to express cFos in RES-LD rats was significantly higher than the proportion activated in NF rats (Figure 10). Within the MRF, however, the difference in GLP-1 activation between NF and RES-LD groups was not significant ($p = 0.64$). Interestingly, significantly larger proportions of cNTS GLP-1 neurons were activated in all four fed groups compared to activation of GLP-1 neurons within the MRF ($p < 0.05$ for each within-feeding group comparison; Figure 10). Among the three groups that consumed unrestricted (i.e., satiating) amounts of EN, diEN, or LD, there were no between-groups differences in activation of GLP-1 neurons within either the cNTS or within the MRF ($p > 0.05$ for each between-group comparison; Figure 10).

An adjacent tissue set from each meal-entrained rat was dual immunolabeled for PrRP and cFos. The proportion of PrRP-positive neurons in cNTS that express cFos is presented for each of the five feeding conditions in Appendix A.

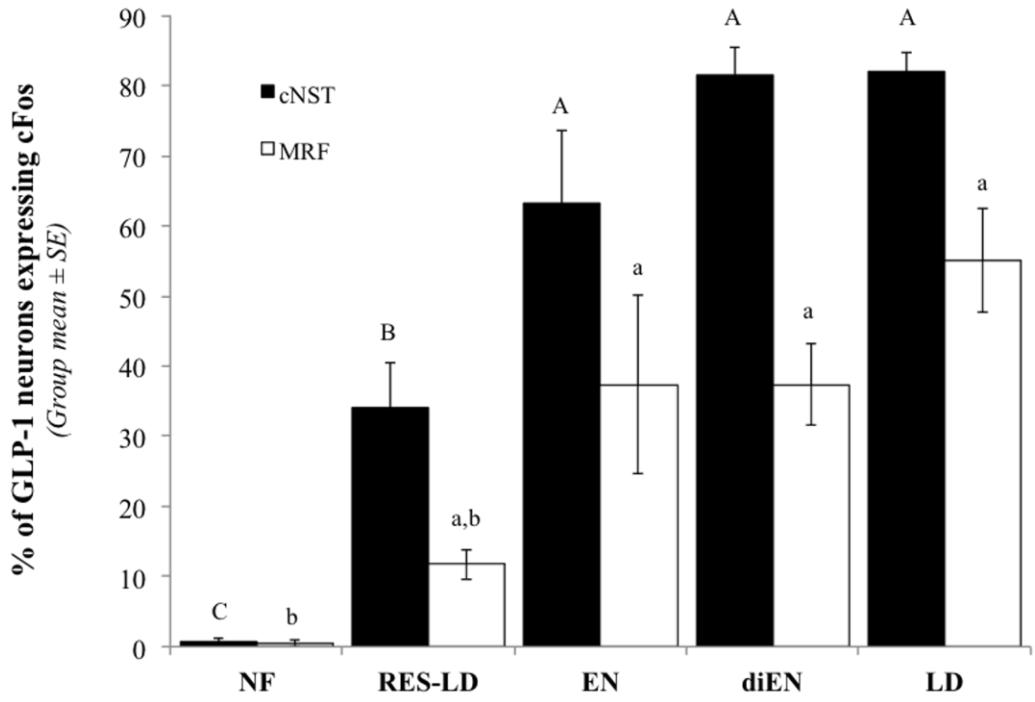

Figure 10. GLP-1 neuronal activation in meal-entrained rats

Percentage of GLP-1-immunopositive neurons that express cFos immunoreactivity in rats consuming meals of different sizes or types (Experiment 1). GLP-1 neurons within the cNTS (black bars) and medullary reticular formation (MRF; open bars) are presented separately. Within each regional subgroup of GLP-1 neurons, different letters indicate significant differences in cFos activation between feeding condition groups (p <.05).

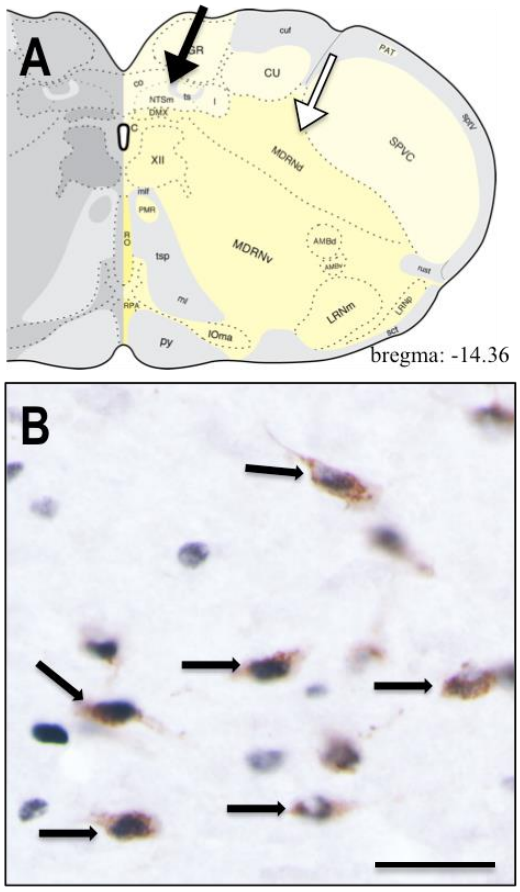

Figure 11. Example of GLP-1 and cFos dual immunolabeling in cNTS

GLP-1-positive (B; brown peroxidase labeling) neurons within the cNTS (region indicated by black arrow in panel A) are activated to express cFos (B; purple/black nuclear labeling) in a representative rat that consumed 14% of its BW in LD (Experiment 1). Black arrows in B point out some of the double-labeled neurons in which nuclear cFos immunolabeling is co-localized with cytoplasmic GLP-1. White arrow in A indicates the MRF, where additional GLP-1-positive

neurons reside. Scale bar in B = 50 μm. Brain schematic image in panel A adapted from (Swanson, 2004).

3.3.1b. Correlational analyses of GLP-1 neuronal activation vs. amount consumed. Figure 12 plots the grams of test diet consumed by individual rats (expressed as % BW) compared to cFos activation of cNTS GLP-1 neurons across all feeding groups in Experiment 1. For purposes of comparison, food intake (as % BW) and GLP-1 cFos activation data from our recently published study (Kreisler et al., 2013, Chapter 3) using non-entrained rats that consumed unrestricted amounts of Ensure also are plotted in Figure 12. In that earlier study, experimentally naïve rats that were fasted overnight and then re-fed for the first time consumed, on average, 4.7% BW of Ensure and displayed cFos activation of ~27.5% of cNTS GLP-1 neurons (Figure 5). Across both studies, activation of cNTS GLP-1 neurons was positively (Pearson's R = 0.86) and significantly ($p < 0.001$) correlated with amount consumed (as % BW) across all 5 experimental fed groups (note that Entrained: NF group data are plotted in Figure 12 but are not included in correlational analysis). When only entrained (refed) rats in the present study are considered, activation of cNTS GLP-1 neurons was also positively (Pearson's R = 0.82) and significantly ($p < 0.001$) correlated with amount consumed (as %BW). An adjacent tissue set from each rat was dual immunolabeled for PrRP and cFos. Correlational analysis between activation of cNTS PrRP neurons and amount consumed is presented in Appendix A.

cFos activation of cNTS and MRF GLP-1 neurons in the present study also was positively and significantly correlated with the calculated value of calories emptied from the stomach (i.e., reaching the intestines; Table 2) by the time of perfusion (Pearson's R = 0.66, $p = 0.001$ for cNTS GLP-1 activation vs. calories emptied; R = 0.60, $p = 0.002$ for MRF GLP-1 activation vs. calories emptied).

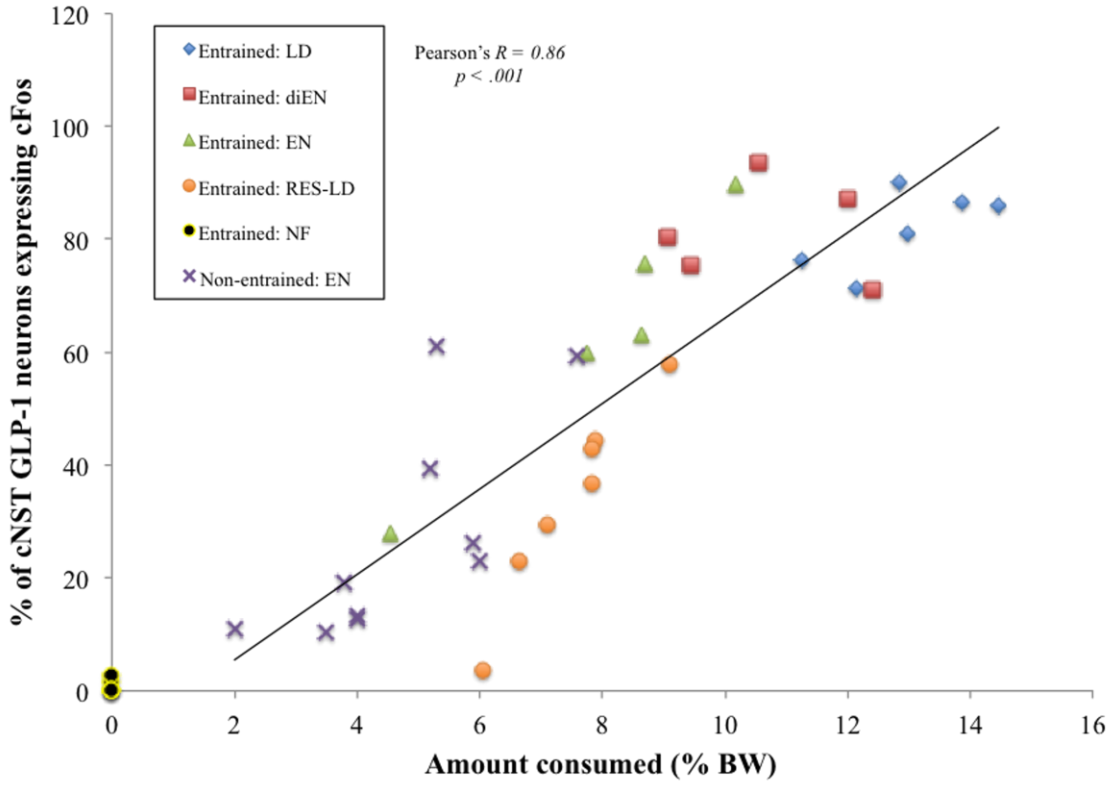

Figure 12. Relationship between amount consumed (as %BW) and cNTS GLP-1 neuronal activation in meal-entrained rats consuming different diets (Experiment 1).

Each symbol represents one rat. Data from non-entrained, Ensure-fed rats ("Non-entrained: EN") from Figure 7 are included for comparison. Data from Entrained: NF rats also are plotted for comparison, but the indicated correlation value is derived only from the five fed groups.

3.3.2 Experiment 2: Ensure intake and meal pattern analyses in meal-entrained rats after central blockade of GLP-1 receptors

Daily 2 h Ensure intake (expressed as kcal and as % BW) increased progressively during the acclimation period, stabilizing by day 4 (Figure 13). Overnight chow intake simultaneously decreased from day 1 to day 3, and then remained stable on days 4-6, resulting in stable total daily caloric intake for the duration of the acclimation period (Figure 13). On average, BW remained stable across the entire acclimation period (322g ± 3.9 on day 1 and 325g ± 4.2 on day 6, data not shown).

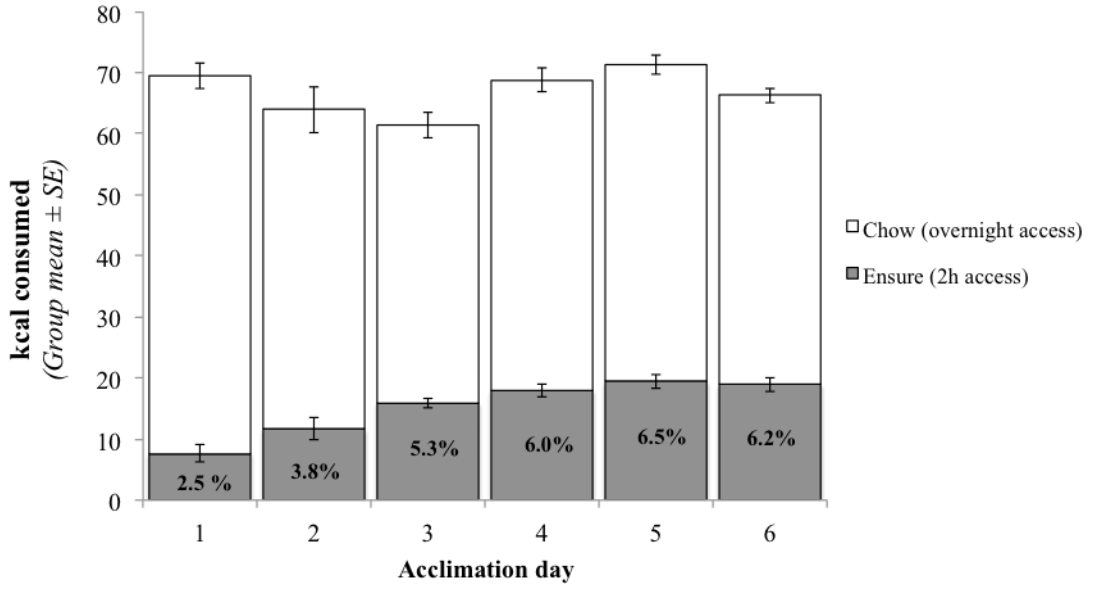

Figure 13. Daily food intake (expressed as kcal) during the meal-entrainment acclimation period for cannulated rats in Experiment 2.

Data from all 13 rats are represented for each day. Paired-samples T-test revealed no significant difference in 2 h Ensure intakes between days 5 and 6 ($p > 0.05$). Experimental treatment (i.c.v. injections) occurred on day 7. The indicated percentage values (inside the gray bars) are the average Ensure intake volume each day, expressed as %BW.

3.3.2a. Two hour volumetric Ensure intake: Cumulative 2 h intake data at baseline (day 6) and after i.c.v. injection (day 7) were expressed in two ways:

Absolute intake (expressed as %BW): After intake stabilization, cumulative 2 h Ensure intake by each rat was assessed at baseline (day 6, n = 13) and after i.c.v. infusion of Ex9 (n = 6) or SAL vehicle (n = 7) (Figure 14 A). There was a significant interaction between day (i.e., baseline or i.c.v. treatment) and injection condition (i.e., SAL or Ex9) [F (1, 11) = 5.99, p < 0.05]. Post-hoc tests revealed that SAL- and Ex9-injected rats consumed similar volumes of Ensure after i.c.v. injection (6.75 %BW ± 0.47 and 6.45 %BW ± 0.12, respectively, p > .05, Figure 14 A). The interaction was driven by an unanticipated significant difference in day 6 baseline intake between rats that were assigned to the i.c.v. SAL group (which consumed 6.95% BW ± 0.25 at baseline) and those that were assigned to the i.c.v. Ex9 group (which consumed 5.12% BW ± 0.45 at baseline) (Figure 14 A).

Normalized intake (expressed as % change from baseline): Compared to their own individual baseline 2 h Ensure intakes, SAL-injected rats decreased their intake by 2.14% ± 7.03, whereas Ex9-injected rats increased their intake by 29.46% ± 8.54 (Figure 14 B). These change from baseline values were significantly different between i.c.v. injection groups (t (11) = -2.89, p < .05).

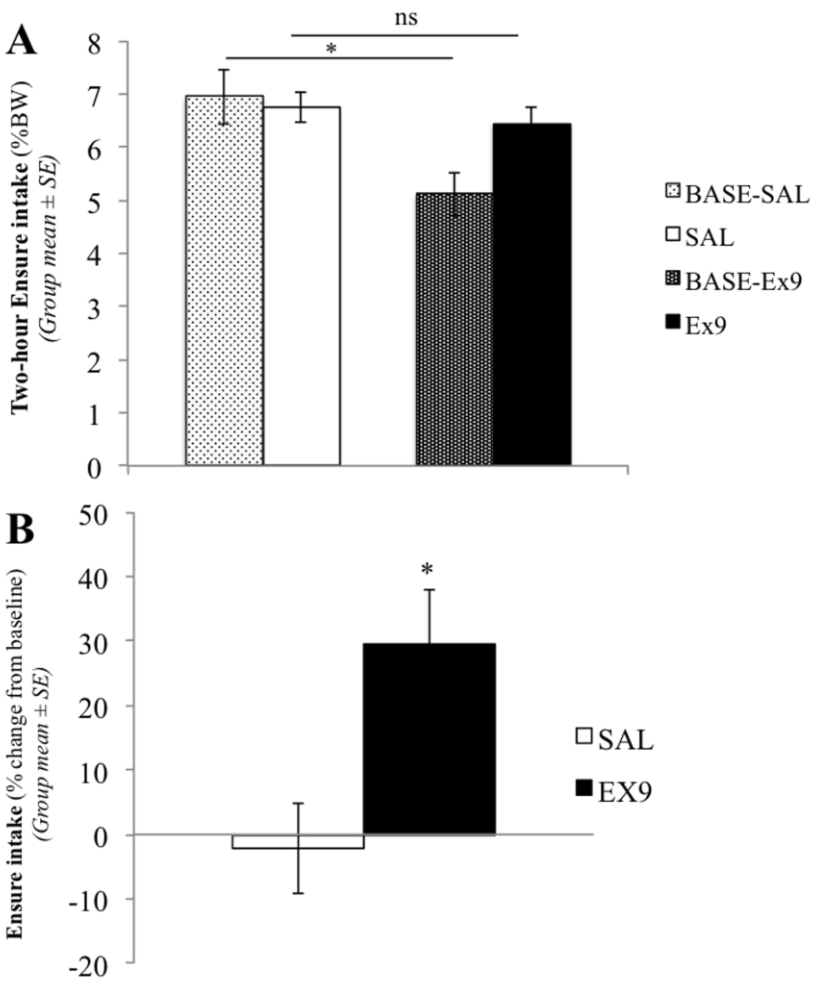

Figure 14. Cumulative 2h Ensure intake in ad lib-fed, meal-entrained rats (Experiment 2). Panel A shows between-groups comparisons of cumulative intake (as %BW) by SAL- and Ex9-injected rats (day 7), and also shows baseline intake by the same rats within each group (day 6; BASE-SAL and BASE-Ex9). In panel B, cumulative 2 h intake on the i.c.v. injection day (day 7) is normalized (within-subjects) to each rat's own baseline intake (day 6), and then averaged within each i.c.v. injection group. *ns,* not significantly different (T-test, $p > .05$); *, significantly different (T-test, $p < .05$).

3.3.2b. Meal structure: Meal structure during each 2 h feeding period was assessed based on lickometer data. Data reflecting meal number, size, duration, and inter-meal interval (IMI) of Ensure intake by rats on baseline and i.c.v. injection days (absolute values, Table 3) also were normalized to each rat's own baseline Ensure intake data (% change from baseline, Table 3).

Absolute values. During the 2 h feeding period, rats typically consumed 2 meals separated by an IMI of approximately 1 h (Table 3), regardless of treatment group or condition. Since every rat consumed at least two meals, but only a few rats consumed a third, comparisons of meal size and duration were applied separately to the first and second meals. ANOVA revealed no significant main effects of day or injection condition on the size or duration of the first meal, on meal number, or on IMI ($p > 0.05$ for each parameter). However, while there also was no main effect of day on any second meal parameters, there was a significant interaction between day and injection condition on both the size [F (1, 11) = 6.32, $p < 0.05$] and duration [F (1, 11) = 6.45, $p < 0.05$] of the second meal. Post-hoc between subjects tests revealed that rats consumed significantly larger second meals after i.c.v. Ex9 (~2990 licks) than after i.c.v. SAL (~1350 licks; $t(11) = -2.40$, $p < 0.05$, Table 3). Paired within-subject comparisons revealed that, for SAL-treated rats, the duration of the second meal after i.c.v. injection (~4.7 min) was significantly reduced compared to the duration of the second meal under baseline conditions (~10.1 min; SAL vs. BASE-SAL values, $t(6) = 2.80$, $p < 0.05$, Table 2). The difference between the size of the second meal within SAL-injected rats after i.c.v. treatment (~1350 licks) compared to their own baseline values (~2939 licks) also trended towards statistical significance ($p = 0.08$). Within the Ex9-treated group, however, there were no significant differences in absolute values for any meal-related parameter assessed on i.c.v. injection day compared to baseline (Table 2).

Normalized values (expressed as % change from baseline): Meal structure data were additionally expressed as the percent change in each parameter's value after i.c.v. injection compared to the same rat's non-injected baseline value, then combined by injection group (bottom two rows, Table 3). Consistent with the comparisons of absolute intake values, the duration of the second meal in SAL rats after i.c.v. injection was reduced by ~46% compared to baseline, whereas the duration of the second meal in Ex9 rats after i.c.v. injection was increased by ~32% compared to baseline. These differential within-subjects effects of i.c.v. treatment were significantly different ($t(11) = -2.23$, $p < .05$, Table 2). Similarly, the size (in licks) of the second meal in SAL rats was reduced by ~40% after i.c.v. injection compared to baseline, whereas the size of the second meal in Ex9 rats was increased by ~47% compared to baseline (bottom two rows, Table 3). These within-subjects effects of i.c.v. treatment also were significantly different ($t(11) = -2.35$, $p < .05$, Table 3). For all other meal parameters analyzed, the change from baseline after i.c.v. SAL vs. Ex9 did not differ between groups ($p > .05$ for all comparisons, Table 3).

Table 3. Meal pattern data in meal-entrained rats

Meal pattern data on baseline (no injection; BASE-SAL, BASE-EX9) and i.c.v. injection days (SAL, EX9) in Experiment 2. The top four rows indicate absolute (i.e., non-normalized) values. Values in the bottom two rows (shaded) were generated by normalizing absolute values for each parameter in rats after i.c.v. injection to their own baseline values for the same parameter (expressed as % change from baseline). *Absolute value significantly greater than respective SAL absolute value; # Absolute value significantly less than respective BASE-SAL value; $ Normalized value after Ex9 significantly greater than respective SAL normalized value (T-tests, *p < .05)*.

Experimental treatment condition	Meal number	Meal 1 size (lick #)	Meal 2 size (lick #)	Meal 1 duration (min)	Meal 2 duration (min)	Inter-meal interval (IMI, min)
BASE-SAL (absolute values)	2.57 ± 0.43	4239.71 ± 493.24	2938.57 ± 426.08	17.29 ± 1.27	**10.14 ± 1.40**	50.00 ± 8.13
SAL (absolute values)	2.43 ± 0.20	5254.43 ± 283.85	**1350.29 ± 405.24**	17.29 ± 1.17	**4.71 ± 1.41#**	55.57 ± 9.95
BASE-EX9 (absolute values)	2.17 ± 0.17	4495.50 ± 592.93	2239.33 ± 474.76	16.67 ± 1.12	6.67 ± 0.99	77.50 ± 9.80
EX9 (absolute values)	2.33 ± 0.33	4997.17 ± 434.24	**2990.17 ± 567.35***	14.50 ± 1.20	8.50 ± 2.08	68.83 ± 10.86
SAL (% change from baseline)	3.81 ± 13.46%	31.84 ± 12.98%	**-40.23 ± 20.51%**	5.13 ± 13.78%	**-46.41 ± 14.86%**	2.02 ± 18.41%
Ex9 (% change from baseline)	11.11 ± 18.59%	23.00 ± 22.13%	**46.99 ± 32.33%$**	-11.32 ± 9.35%	**32.26 ± 34.21%$**	34.73 ± 44.05%

3.4 DISCUSSION

The present study was designed to investigate feeding-induced recruitment of hindbrain GLP-1 neurons in rats entrained to consume very large volumes of liquid diet, and also to explore a role for central GLP-1 signaling in controlling food intake under such conditions. Our cFos results indicate that GLP-1 neurons are progressively recruited in rats consuming progressively larger volumes of liquid diet. Similar volumes of liquid diet were consumed by rats after i.c.v. injection of SAL or Ex9 (between-subjects comparison). However, when intake after i.c.v. injection was compared to intake by the same rats under non-injected baseline conditions (within-subjects comparison), Ex9 treatment increased cumulative 2 h intake, whereas SAL treatment had no significant effect on cumulative intake. Additional insights into the effects of i.c.v. Ex9 or SAL on intake were gained from meal pattern analysis, which revealed that increased cumulative intake after Ex9 treatment was the result of larger second meals (# of licks) during the latter portion of the 2h feeding period. Thus, our results suggest that GLP-1 receptor signaling helps to limit meal size after a substantial amount of food has already been consumed. In addition, however, i.c.v. SAL treatment alone reduced the duration of the second meal by nearly 50% compared to non-treated baseline conditions. These results highlight the importance of considering both cumulative intake and meal pattern data collected under both non-manipulated control and experimental conditions, particularly when assessing the role of stress-sensitive neuropeptide systems such as GLP-1 (Maniscalco et al., 2012b) in controlling food intake and other motivated behaviors.

3.4.1. Feeding-induced activation of GLP-1 neurons: cNTS.

In Experiment 1, 60-80% of GLP-1 neurons in the cNTS were activated to express cFos after rats ate to satiety by consuming 7-13% of their BW in liquid diet during the allotted 1h feeding period (Figure 12). This level of GLP-1 activation was 2-3 times higher than in our previous report (Kreisler et al., 2013) in which rats were not acclimated to consuming large meals. After an overnight fast, rats in that study consumed only 5% BW of Ensure, and only ~30% of GLP-1 neurons were activated (Kreisler et al., 2013). In the present study, there were no differences in GLP-1 activation between rats consuming the entrained LD vs. the relatively more novel Ensure, regardless of dilution, suggesting that neither caloric density, novelty, nor flavor/palatability contributed significantly to feeding-induced GLP-1 neuronal activation. In a previous study, Vrang et al. (Vrang et al., 2003) reported that ~30-40% of GLP-2-positive neurons (i.e., which also are GLP-1-positive) were activated to express cFos in rats after inflation of a gastric balloon with 9 ml of saline, similar to the volume of Ensure voluntarily consumed by non-entrained rats in our previous study (Kreisler et al., 2013). Thus, GLP-1 neuronal activation likely is due to stimulation of vagal mechanoreceptors that are sensitive to gastric distension. Indeed, correlational analyses in the present study indicated that cNTS GLP-1 neural activation was best predicted by the volume ingested, although we cannot rule out a possible additional contribution of intestinal nutrient exposure.

Despite eating more voluminous meals and emptying more calories into the intestines, RES-LD rats in the present study consumed ~19 ml (~7.5% BW) but displayed similar proportions of cNTS GLP-1 neuronal activation (~35%) as non-entrained, satiated rats in our previous study (~30% activation) (Kreisler et al., 2013), although rats in the previous study

consumed only ~9 ml (~5% BW) (Kreisler et al., 2013). Considered together, these results suggest that the ability of a given amount of feeding-induced gastric distension to activate cNTS GLP-1 neurons varies based on experience consuming large meals. Specifically, meal entrainment may shift the intake-response curve to the right, such that greater amounts of food must be consumed in order to recruit the same proportions of GLP-1 neurons that are recruited by smaller amounts of food intake in non-entrained rats. For a given volume of intake and gastric load, the reduced levels of GLP-1 activation in meal-entrained RES-LD rats (present study) vs. non-entrained rats in our previous study (Kreisler et al., 2013) may reflect physiological adjustments that permit larger meals to be consumed after entrainment (Woods, 1991; Woods and Ramsay, 2000; Woods, 2002).

3.4.2. Feeding-induced activation of GLP-1 neurons: MRF.

Interestingly, although approximately half of all identified GLP-1 neurons in the caudal brainstem reside in the MRF, GLP-1 neurons within the cNTS were more sensitive to feeding-induced activation, consistent with a previous report (Gaykema et al., 2009). These GLP-1 regional differences in cFos activation may be due to the fact that the cNTS receives direct vagal sensory input, including input arising from the GI tract, whereas sensory inputs to the MRF are indirect. The neural pathway(s) through which MRF GLP-1 neurons are recruited in rats after food intake is unknown. However, given that MRF neurons are recruited in an intake volume-dependent manner, similar to the progressive recruitment of cNTS GLP-1 neurons (Figure 12), the recruitment of MRF GLP-1 neurons could reflect relayed input from the cNTS (Aicher et al., 1995; Bailey et al., 2006).

3.4.3. GLP-1 receptor blockade in meal-entrained rats.

Our cFos data provide convincing evidence that GLP-1 neuronal activation closely reflects the amount consumed, in a manner that is modified by feeding experience (i.e., meal entrainment). Importantly, however, rats must consume at least 5-6% of their BW within a 1 h period (achieved either after fasting or by meal entrainment) before GLP-1 neural activation increases above baseline activation levels measured in rats with *ad lib* food access (Maniscalco and Rinaman, 2013). Therefore, insofar as cFos is an indicator of GLP-1 neural activation, our results indicate that GLP-1 neurons are relatively insensitive to sensory feedback signals generated by food intake, except when rats consume unusually large amounts during a relatively short period of time. It follows that central blockade of GLP-1 receptor signaling should have little impact on food intake when endogenous GLP-1 signaling is relatively low, whereas an effect may be revealed when increased GLP-1 signaling accompanies the consumption of very large meals. Indeed, several studies have reported that i.c.v. Ex9 does not increase chow intake in *ad lib*-fed rats compared to intake by control rats treated with i.c.v. vehicle (Grill et al., 2004; Kanoski et al., 2011; Alhadeff et al., 2012; Zhao et al., 2012).

The new Ex9 experiment in the present study used *ad-lib* fed rats that were trained to consume large volumes of palatable Ensure within a 2 h period. According to our hypothesis, this is the type of feeding scenario in which endogenous GLP-1 signaling may contribute to the termination of intake. If so, then central administration of Ex9 should block such signaling, promoting more excessive intake. Cumulative 2 h intake in rats after i.c.v. Ex9 was compared both to baseline intake by the same rats in the absence of injection (i.e., within subjects) and also

to intake by a different group of rats after i.c.v. SAL treatment (i.e., between subjects). Ex9 significantly increased 2 h cumulative intake within subjects, but not when compared to 2 h intake by rats treated with i.c.v. SAL. Meal structure analyses revealed that, compared to the within-subjects effects of i.c.v. SAL treatment, Ex9 treatment increased the size and duration of the second (but not the first) meal consumed compared to baseline values in the same rats, and also increased the size of the second meal compared to values in SAL-treated rats. The differential effect of Ex9 vs. SAL treatment on second meal size was further enhanced by a significant decrease in second meal size in SAL-treated rats compared to their own baseline, likely reflecting a delayed hypophagic response to the mild stress of handling and i.c.v. injection that was not apparent during the first meal.

The current analysis of cumulative intake and meal pattern data in rats under both non-injected control conditions and after experimental treatment provides novel insights into the potential role of endogenous GLP-1 signaling in regulating food intake. To our knowledge, only one previous study has reported the effect of central Ex9 treatment on meal structure, with no reported effects on cumulative intake (Dossat et al., 2013). In that study, *ad lib*-fed rats were trained to consume sweetened condensed milk or sucrose solution for 2 h each day, and then received either vehicle or Ex9 (in counterbalanced order) infused unilaterally into the NAcc before test meals. Compared to intake after vehicle infusion, Ex9 increased meal size and duration and altered licking microstructure in a manner consistent with increased palatability, suggesting a site-specific role for GLP-1 signaling within the NAcc to reduce food palatability (Dossat et al., 2013). However, meal structure data under non-injected baseline conditions were not reported, leaving open the possibility that intra-NAcc Ex9 also exerts at least some of its

effects by attenuating or counteracting the hypophagic effect of experimental treatment (i.e., central infusion).

The effect of Ex9 to increase food intake/meal size in rats consuming 6-7 % of their BW (Experiment 2) is consistent with a substantial recruitment (40-50%) of GLP-1 neurons after rats consume similar volumes of liquid diet (Experiment 1). Thus, a behavioral effect of blocking endogenous GLP-1 signaling can be revealed when significant proportions of GLP-1 neurons are active. However, in considering the potential role of hindbrain GLP-1 neurons, it is important to remember that GLP-1 is not their sole signaling molecule. In rats, GLP-1 neurons are glutamatergic (Zheng et al., 2014) and are likely to express a variety of additional neuropeptides, including inhibin β, enkephalin, and/or somatostatin (Sawchenko et al., 1990). Thus, the relatively subtle effect of pharmacological GLP-1 receptor antagonism on cumulative food intake and meal size under certain experimental conditions does not preclude a potentially more important and/or more physiological role of GLP-1 neurons to control food intake that involves signaling by one or more co-expressed transmitter molecules.

3.4.4. Perspectives

We interpret our results as evidence that central GLP-1 signaling plays a role in satiation in rats consuming large, entrained meals of liquid diet. Compared to intake under baseline (i.e., non-injected) conditions, i.c.v. administration of Ex9 increased cumulative intake volume, which was driven by an increase in second meal size in Ex9-treated rats compared to a decrease in vehicle-treated rats. Since i.c.v. saline vehicle reduced meal size and duration compared to intake under non-injected baseline conditions, at least part of the observed effect of Ex9 treatment is likely

attributable to drug-induced attenuation of the hypophagic effects of stress produced by the handling and i.c.v. injection procedures. Indeed, central administration of Ex9 attenuates the hypophagic effects of other stressful treatments, including systemically administered LiCl (Rinaman, 1999b) or endotoxin (Langhans et al., 1991; Grill et al., 2004). Although previous studies have reported that rats consume more food after i.c.v. Ex9 than after i.c.v. vehicle, these studies did not report baseline food intake under non-injected control conditions (Hayes et al., 2009; Kanoski et al., 2011; Alhadeff et al., 2012; Zhao et al., 2012; Dossat et al., 2013). Therefore, while our results are consistent with those earlier reports, the current findings provide additional insight into the role of endogenous GLP-1 signaling in controlling food intake, i.e., by reducing meal size near the end of the feeding period when meal-entrained rats are consuming unusually large amounts of food, and also under experimental conditions that promote stress-induced hypophagia.

4.0 GENERAL DISCUSSION

4.1 SUMMARY AND INTERPRETATION

Satiation is a sensory-driven process that begins when nutrients make contact with the mouth. While it is clear that the hindbrain is a hub in the central network involved in satiation, the neural phenotypes that participate in the integration of feeding-related sensory feedback and subsequently inhibit the motor patterns involved in feeding are unknown.

GLP-1 and PrRP-positive neurons in the caudal hindbrain are sensitive to a wide array of interoceptive stimuli, including those that reduce food intake. Furthermore, loss-of-function studies have supported a causal role for both systems in the regulation of energy balance. However, whether they are involved in the control of satiation is less clear. We proposed that these two signaling systems play a role in the control of physiological food intake. To challenge this hypothesis, my dissertation work was designed to address two overarching Aims:

Aim 1: Demonstrate that food intake itself (in the absence of other experimental manipulations) activates DBH-positive, including PrRP-positive, and GLP-1-expressing neurons and assess the feeding-related sensory signals that recruit them.

Aim 2: Assess the contribution of endogenous GLP-1 signaling to the control of food intake

In Chapter 3, we quantified cFos expression (a marker of neuronal activation) among phenotypically-identified DBH-positive (including both PrRP-positive and -negative subsets) and GLP-1 neurons in rats that undergoing their first experience with food deprivation following by refeeding on meals of differing sizes. Results demonstrated that:

1. Both DBH-positive A2 and GLP-1 neurons effectively track food intake when fasted rats consumed satiating meals of varying sizes.

2. Among DBH-positive neurons, those that express PrRP were more sensitive to meals of all sizes than those that do not express PrRP.

3. Neuronal activation among PrRP-positive and GLP-1 neurons after large satiating meals (5 %BW) was significantly more than the proportion of activation observed after non-satiating meals.

Overall, these results support our overarching hypothesis that DBH-positive A2 and GLP-1-positive neurons are sensitive to feeding-generated sensory signals. However, even the largest satiating meals consumed by these non-entrained rats activated only ~30%, and 45% of GLP-1 and PrRP-positive neurons, respectively. Since the proportion of GLP-1 and PrRP-positive neurons expressing cFos was significantly and positively correlated with intake volume (Figures 7 and 8), we hypothesized that rats consuming even larger volumes would display proportionately higher levels of GLP-1 and PrRP neural activation. To challenge this hypothesis,

quantification of cFos expression among the PrRP and GLP-1-positive neurons was extended to rats that were trained over 5 days to consume significantly larger volumes within a 1 h period (Chapter 4). Because activation of PrRP-negative neurons was significantly less than that of PrRP-positive neurons across all intake volumes in the previous experiment, quantification of cFos expression within the PrRP-negative subpopulation of A2 neurons (of which there are relatively few) was not performed in the following cFos experiment (Chapter 4). Results of cFos experiments in Chapter 4 demonstrated:

1. Larger entrained meals produced a more robust proportion of activation among both GLP-1-positive and PrRP-positive populations that similarly correlated with intake volume and caloric value (Figures 12 and 17).

2. Entrained rats that consumed non-satiating meals, showed lower proportions GLP-1 and PrRP activation than would be expected based on the absolute volume of the those meals.

3. Neuronal activation was not obviously affected by flavor nor novelty of the diet.

4. Despite the fact that half of all hindbrain GLP-1 neurons reside in the MRF, this subpopulation was less sensitive to food intake that those in the cNTS.

The progressive increase in GLP-1/ PrRP neuronal activation assessed in rats after intake of progressively larger volumes suggests that both populations of neurons participate in the process of satiation. However, it also is possible that these populations of neurons are simply

tracking gastric distension or some other feeding-related sensory signal, without actively participating in meal size control. To explore this, we used a commercially available specific GLP-1 receptor antagonist to block endogenous GLP-1 signaling and assessed subsequent meal-related parameters and cumulative intake. Unfortunately there is no antagonist for GPR10 that would allow a comparable study to be performed for endogenous PrRP signaling. An alternative and novel approach is being developed in our laboratory that will enable loss-of-function studies to be performed for endogenous PrRP signaling specifically (Section 6.0).

Results of pharmacological studies in Chapter 5 demonstrated that:

1. Blockade of GLP-1 receptors increased cumulative intake volume compared to the amount consumed by the same animals in the absence of injection (baseline).

2. Compared to baseline, saline injection decreased the size and duration of meals consumed during the latter half of the feeding period, whereas Ex9 treatment increased both of these parameters.

3. Neither Ex9, nor SAL treatment had an effect on indices of satiety (intermeal interval and meal number), nor on the size/duration of meals consumed during the initial minutes of the feeding period.

Behavioral results of Chapter 4 supported a causal role for GLP-1 signaling in physiological satiation and not satiety during entrained meals. Such a role includes the ability to attenuate hypophagic responses to the mild stress associated with saline injection.

4.2 DIFFERENTIAL ACTIVATION: PRRP V. GLP-1

Assessing cFos expression in both PrRP-positive and GLP-1 neurons in the same animal allows for a direct comparison between the sensitivities of these two neuronal populations to meals of various sizes. Interpreted in and of itself, the pattern of GLP-1 activation in non-entrained rats (Figure 5) suggests that satiation is required for activation of GLP-1 neurons above the levels of fasted animals. However, when considering the progressive nature of GLP-1 neural activation responses that becomes apparent across a broader range of intakes (Figure 12), perhaps intake of 2-3 %BW (consumed by 50% and 30% restricted groups, Figure 5) simply fails to reach a threshold required to begin recruiting GLP-1 neurons. However, meals of 3 %BW (30% restricted group) *were* sufficient to begin recruiting A2 (both PrRP-positive and PrRP-negative) neurons above fasting levels. In other words, differential sensitivities to feeding-generated sensory signals between A2 and GLP-1 neurons were particularly apparent when meal sizes were relatively small (less than ~ 4% BW).

This differential sensitivity between PrRP-positive and GLP-1 neurons was also apparent in rats that consumed restricted amounts of the expected LD (RES-LD). This group provided important information about the degree to which expectation or previous experience with very large meals influences neuronal responses. RES-LD rats ate non-satiating meals (60% of what they were used to eating), but the absolute volume of these meals (~7.5 %BW) and the amount

that emptied into the intestines (~7 kcal) were still larger than those of non-entrained Ensure meals consumed by non-entrained rats (Table 1). Interestingly, in RES-LD rats, the proportion of activation among both GLP-1 neurons and PrRP-positive was below the expected proportion based on the intake-response relationship among satiated rats (represented in Figures 12 and 17). The suppression was particularly apparent for PrRP-positive neurons (Appendix B, Figure 17). This result suggests that experience with cycles of deprivation followed by large meals (i.e., meal-entrainment) shifts the intake-response curve to the right, such that PrRP-positive and GLP-1 neurons are less sensitive to a gastric load of a given size.

The biological mechanism that is responsible for such entrainment-associated plasticity can only be speculated upon. One conceivable mechanism may be desensitization of vagal afferent neurons that carry feeding-generated sensory signals from the GI tract and synapse in the cNTS. The effect of meal-entrainment per se on the ability of vagal afferent to recruit brainstem neural circuits has never been directly explored. However, the sensitivity of vagal afferent neurons to metabolic signals is suppressed by diet-induced obesity (de Lartigue et al., 2011b; de Lartigue et al., 2011a; de Lartigue et al., 2012), suggesting that feeding experience may alter vagal function.

Another possibility may be the learned anticipatory hormonal (e.g., increased systemic ghrelin and insulin) or visceral motor (gastric relaxation) adjustments that have been previously described (Woods, 1991; Woods and Ramsay, 2000). From an adaptive perspective, these adjustments serve to permit larger meals during scenarios when food is scarce and must be consumed in large quantities in a confined period of time (replicated experimentally using meal-entrainment). The relationship between such physiological adjustments and PrRP/GLP-1 neurons has not been specifically investigated. However, it is reasonable to hypothesize that these

adjustments involve the quieting of neural circuits with a presumed role in inhibiting food intake such as those that involve PrRP and/or GLP-1 neurons, in order to permit large meals. Viewed from this perspective, the suppression of feeding-induced neuronal activation amongst PrRP and GLP-1 neurons might itself be such an adjustment.

Although the proportions of both cNTS GLP-1 and PrRP-positive neurons that are activated by feeding closely reflect the amount consumed, the relationship between meal size and proportion of activation differs slightly between the two populations. In rats that consumed relatively smaller volumes (0-6 %BW), PrRP activation tended to be higher than that of GLP-1. In contrast, among rats that ate much larger amounts (~9-14 %BW), GLP-1 neuronal activation was usually higher than that of PrRP-positive neurons (Figure 15). Neither the anatomical nor functional relationship between PrRP and GLP-1-positive neurons has been explored. Also, cFos expression does not offer information about strength nor timing of synaptic input onto these neurons, nor firing patterns. Therefore, it is difficult to speculate about if or how these results reflect a certain degree of intercommunication between these populations.

However, a sensible interpretation of these differential sensitivities is that PrRP and GLP-1-positive neurons might participate in parallel pathways that each transduces feeding-related information to central feeding networks in a slightly different way. Such a scenario might have adaptive significance in that, due to their slightly different intake-response patterns, together PrRP and GLP-1-postive neurons can provide more complete information about the meal.

Another interpretation of this inter-phenotype comparison is that the PrRP and GLP-1 neurons that were activated by smaller meals represent a distinct subset from those that are activated by larger meals, and the relative size of these subsets differs between PrRP and GLP-1 neurons. The extent to which subpopulations of PrRP or GLP-1 neurons have distinct projection

targets or carry out distinct functional roles has not been adequately explored. However, some evidence exists for functionally diverse GLP-1 receptor populations (Kinzig et al., 2002; Kinzig et al., 2003) that may receive input from distinct subsets of cNTS neurons (Vrang et al., 2007).

Despite slight variations in the progression of activation, GLP-1 and PrRP-positive neurons both seem to reach a maximal proportion of activation, 60% for PrRP-positive neurons and 85% for GLP-1-positive neurons, at meals that were ~9 %BW (i.e., rats that consumed 14 %BW showed no higher activation than those that consumed 9 %BW, Figure 15). Since further (> 14 %BW) intake is unlikely under voluntary conditions (Figure 9), this is likely to be the maximal proportion of each of these groups of neurons that are recruited during physiological feeding. Furthermore, if GLP-1 and PrRP-positive neurons do play a causal role in satiation, then presumably 85% or 60% of the population is sufficient to do so.

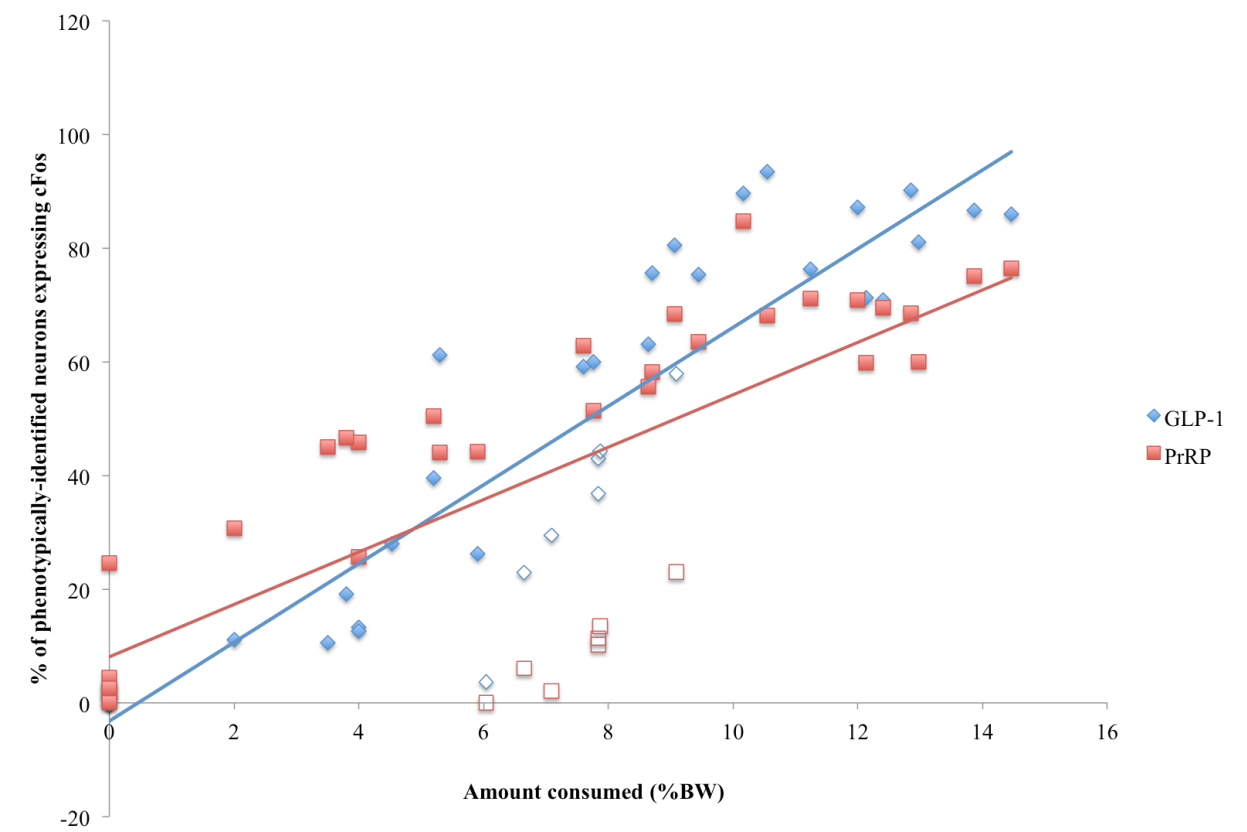

Figure 15. Differential activation of cNTS GLP-1 and PrRP-positive neurons

Composite data depicting the relationship between meal size and percentage of GLP-1-positive and PrRP-positive neurons activated to express cFos in entrained and non-entrained rats (data compiled from Figures 7, 8, 12, and 16) under various experimental conditions. Each rat is represented by one blue and one red symbol that lie at the same horizontal coordinate. Feeding condition is not depicted for clarity. Open symbols represent RES-LD rats (Chapter 4) for which the indicated volume consumed was not satiating.

4.3 SATIATION VS. STRESS-INDUCED HYPOPHAGIA: IMPORTANCE OF INCLUDING NON-MANIPULATED CONTROLS

Food intake is a normal daily process. However, food intake disturbs many tightly regulated physiological parameters and can itself be considered a challenge to homeostasis (Woods, 1991, 2002). Therefore, a fine line exists between day-to-day satiation and stress-induced hypophagia and it is not unreasonable to view these processes as two behavioral phenomena that lie at different points along a continuum of homeostatic disruption. Viewed from this perspective, results of experiments aimed at exploring the neural correlates of feeding behavior must be interpreted with consideration of the experimental, environmental, and physiological context surrounding ingestive event(s).

In the present study, vehicle treatment alone suppressed meal size and duration compared to baseline (non-injected) values. Recently published data from our laboratory demonstrated that an intraventricular saline injection alone activates ~60% of cNTS GLP-1 neurons in *adlib*-fed rats, significantly more than that observed in non-injected *ad lib-fed* controls (~20%, Maniscalco et al., 2015). Taken together, these results support the interpretation that Ex9-associated hyperphagia (compared to intake in vehicle-treated rats) observed in previous studies may be, at least partially, due to an attenuation of enhanced satiation mediated by endogenous GLP-1 signaling after injection procedures. This interpretation is consistent with a role for endogenous GLP-1 signaling in stress-induced hypohagia rather than food intake *per se* (Appendix B).

In order to discriminate between a role for endogenous GLP-1 signaling in stress-induced hypophagia from that of normal satiation, we included intake data from the same rats before any injection procedures had occurred. By comparing these data to those after vehicle and Ex9 treatment, a more complete assessment of the role for endogenous GLP-1 signaling in feeding is

possible. Until now, no existing studies utilizing Ex9 (except Maniscalco et al., 2015, in paragraph above) provide data from non-manipulated/injected controls. Therefore, further experiments that include a "no manipulation" experimental condition are needed in order to gain a better understanding of a role for GLP-1 signaling in normal satiation vs. stress-induced hypophagia.

4.4 INTERPRETING DISCREPANCIES BETWEEN PUBLISHED EX9 STUDIES: CONSIDERING PRESENT RESULTS

A substantial number of studies has measured cumulative food intake in rats after central administration of Ex9 in order to challenge a role for endogenous GLP-1 signaling in feeding. Some of these studies have reported increased food intake associated with Ex9 treatment, while others have reported similar intakes as vehicle injected controls. Together with results of cFos experiments conducted in our lab, closer examination of the different feeding paradigms used in these studies reveals a possible explanation for this discrepancy.

Ex9 treatment fails to have an effect in rats that are fasted overnight and are then allowed to consume a satiating test meal (Turton et al., 1996; Schick et al., 2003a; Hayes et al., 2009). It is presumed that rats in these studies have not been acclimated to fasting and refeeding (meal-entrained) prior to experimental day, although this information is poorly reported. cFos results presented in Chapters 3 and 4 and (Maniscalco and Rinaman, 2013) show that an acute fast silences GLP-1 neurons. Even though consuming a satiating meal after such a fast increases GLP-1 neuronal activation, it only activates ~30% of GLP-1 neurons (Figure 10). Therefore, considering results of these cFos studies, it is not surprising that an overnight fasting followed by

refeeding fails to invoke sufficient GLP-1 signaling to see an effect of blocking it. A direct comparison between the ability of GLP-1 receptor blockade to enhance fasting- versus palatability-driven food intake has not been performed (see Section 6.0, #2, below).

On the other hand, several studies have reported Ex9-associated hyperphagia during experimental procedures that themselves might boost GLP-1 signaling. For example, when food access follows a large Ensure preload, distension of a gastric balloon (Hayes et al., 2009), or a saline injection (Turton et al., 1996; Hayes et al., 2009; Williams et al., 2009; Alhadeff et al., 2012), Ex9 is able to further increase the amount consumed during the experimental food access period. All of these treatments have previously been shown to increase GLP-1 neuronal activation (Vrang et al., 2003; Kreisler et al., 2013; Maniscalco, in press). Therefore, presumably those treatments combined with the feeding that follows them, generates sufficient GLP-1 signaling such that blocking it further increased food intake. Other studies have reported central Ex9-associated hyperphagia in *ad lib*-fed rats during consumption of a palatable test diet (Williams et al., 2009; Alhadeff et al., 2012). Although these studies report neither the amount of maintenance chow nor test diet that is typically consumed in the absence of experimental intervention, it is conceivable that unrestricted intake of a palatable diet in calorically-replete rats produces an unusual degree of gastric distension and thus further boosts GLP-1 signaling. Therefore, Ex9-associated hyperphagia is not surprising under the experimental conditions used in those studies.

5.0 FUTURE DIRECTIONS

Results of studies comprising this dissertation support a role for GLP-1 and PrRP-positive neurons in the caudal NTS in transducing meal-related sensory information to central networks that control meal size. However, our current understanding of the neural circuitry that governs satiation is incomplete. Below is a non-comprehensive list of immediate research directions that address important questions generated from research herein, and that would expand our knowledge surrounding the neural control of satiation.

1. In Chapter 4, we demonstrated that activation of central GLP-1 receptors by endogenous GLP-1 contributes to meal size control. However, that study used a high dose of Ex9 that was administered into the lateral ventricle and presumably accessed all of the diffuse populations of GLP-1 receptors that have been identified (see Section 2.3). Therefore, we cannot attribute the observed hyperphagic effect of Ex9 to any given population in particular. Because the hindbrain is sufficient for satiation in decerebrate rats, GLP-1 receptors within the DVC or medullary nuclei are particularly good candidates for the downstream targets of the activated neurons observed in our studies. However, the possibility that our Ex9 treatment is disrupting an important modulatory role for GLP-1 signaling in forebrain, hypothalamic, or limbic structures cannot be discounted.

2. Pharmacological experiments in Chapter 4 were performed on rats that were *ad lib* fed (on chow) and had not lost BW over the course of the acclimation period. Therefore, Ensure intake in that study was driven by palatability and occurred in excess of homeostatic need. Because we did not perform a similar experiment using Ex9 in fasted rats, we cannot be sure that the observed effect of Ex9 does not extend to homeostatically-driven feeding. Several studies cited in Section 5.4 demonstrated a failure of Ex9 treatment to further increase cumulative food intake in fasted rats, suggesting that a role for endogenous GLP-1 signaling depends on feeding scenario. However, these studies reported neither the effects of such treatment on meal size versus meal frequency, nor included data from non-injected controls. Quantifying 2h fasting-induced cumulative Ensure intake and meal patterns in Ex9 and vehicle-injected rats in our hands would provide an important and direct comparison to those of meal-entrained rats performed in Chapter 4. Together these results would create a more complete picture of the role that endogenous GLP-1 signaling plays in feeding.

3. Results of cFos experiments presented in Chapter 3 and Appendix A support PrRP-positive neurons as another cell group within the cNTS that may play an important role in satiation. Unfortunately, no antagonist for GPR10 is currently available, which limits the ability to explore a causal role for endogenous PrRP signaling in satiation. However, our lab is currently developing a novel lentiviral-mediated short-hairpin RNA vector targeted to PrRP mRNA that will offer a minimally invasive technique by which to eliminate endogenous PrRP signaling specifically. By administering this vector into the PrRP-rich region of cNTS, the contribution of PrRP signaling originating from that region can be revealed. In vivo verification of this shRNA vector is ongoing in our laboratory.

APPENDIX A

ACTIVATION OF PRRP-POSITIVE NEURONS IN CNTS IN MEAL ENTRAINED RATS

Using the same immunolabeling, quantification, and data analysis procedures described in Sections *3.3.3 - 3.3.5*, an adjacent tissue set was used to assess neuronal activation of PrRP-positive neurons from the same meal-entrained rats in Chapter 4 (Experiment 1). Because analyses conducted in Chapter 3 revealed that PrRP-positive neurons are much more sensitive than PrRP-negative neurons to first-time deprivation-induced food intake (Figure 6), PrRP-negative neurons were not included in this analysis in meal-entrained rats.

In meal-entrained rats, as expected, there was no main effect of feeding condition on the number of PrRP-positive neurons counted within the cNTS [161.50 ± 41.67 neurons per rat; between-groups comparison $F(4, 23) = 0.83$, $p > 0.05$]. Also as expected, very few PrRP-positive neurons were cFos-positive in NF rats, whereas cFos expression by PrRP neurons was increased in rats within all three fed groups (EN, diEN, and LD) that consumed satiating meals (Figure 16). ANOVA revealed a significant main effect of feeding condition on the proportion of PrRP-positive neurons expressing cFos within the cNTS [$F(4, 23) = 79.29$, $p < 0.001$]. Interestingly, the proportion of activated PrRP-positive neurons in RES-LD rats (~9.5%) was

statistically similar to that of NF rats (5.9%, $p > 0.05$, Fig 16). Among the three groups that consumed unrestricted (i.e., satiating) amounts of EN, diEN, or LD, there were no between-groups differences in activation of PrRP neurons ($p > 0.05$ for each between-group comparison; Figure 16).

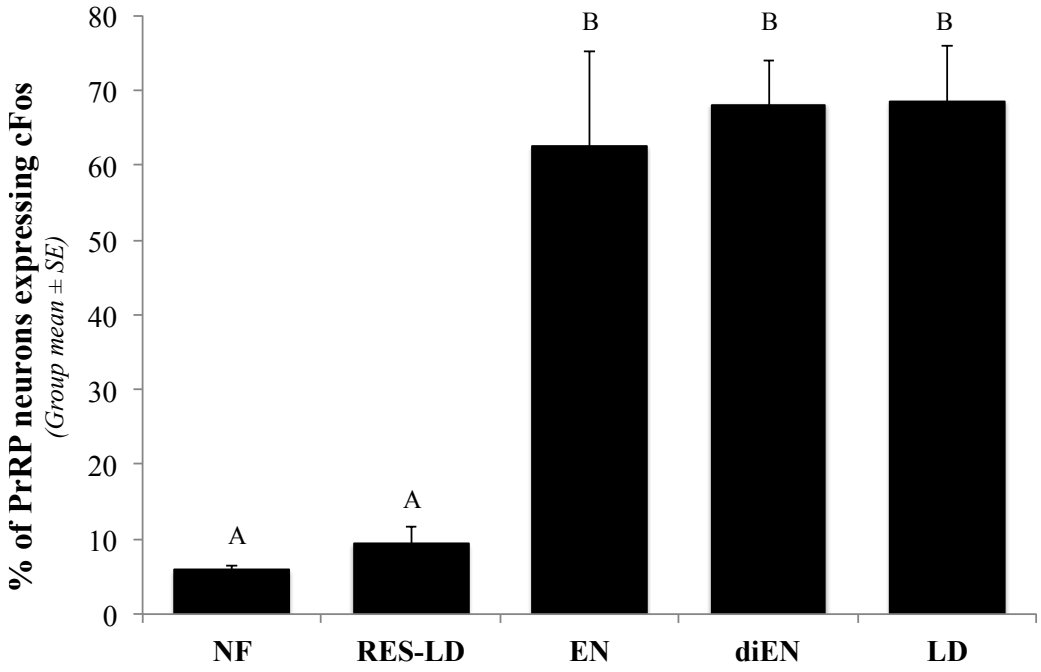

Figure 16. Activation of PrRP-positive neurons in meal-entrained rats

Percentage of PrRP-positive neurons within the cNTS that express cFos immunoreactivity in the same meal-entrained rats used in Chapter 4 (Experiment 1). Different letters indicate significant differences in cFos activation between feeding condition groups ($p < 0.05$).

Figure 17 plots the grams of test diet consumed by individual rats (expressed as % BW) compared to cFos activation of cNTS PrRP-positive neurons across all feeding groups in Chapter 4 (Experiment 1). For purposes of comparison, food intake (as % BW) and PrRP cFos activation data using non-entrained rats that consumed unrestricted amounts of Ensure (Figure 5) also are plotted in Figure 17. In that earlier study, experimentally naïve rats that were fasted overnight and then re-fed for the first time consumed, on average, 4.7% BW of Ensure and displayed cFos activation of ~43.9% of cNTS PrRP neurons (Figure 5). Across both studies, activation of cNTS PrRP-positive neurons was positively (Pearson's R = 0.58) and significantly ($p < 0.002$) correlated with amount consumed (as % BW) across all 5 experimental fed groups (note that Entrained: NF group data are plotted in Figure 17 but are not included in correlational analysis). When only entrained (refed) rats in the present study are considered, activation of cNTS PrRP-positive neurons was also positively (Pearson's R = 0.78) and significantly ($p < 0.001$) correlated with amount consumed (as %BW). cFos activation cNTS PrRP neurons in the present study also was positively and significantly correlated with the calculated value of calories emptied from the stomach (i.e., reaching the intestines; Table 2) by the time of perfusion (Pearson's R = 0.61, $p = 0.003$, not shown).

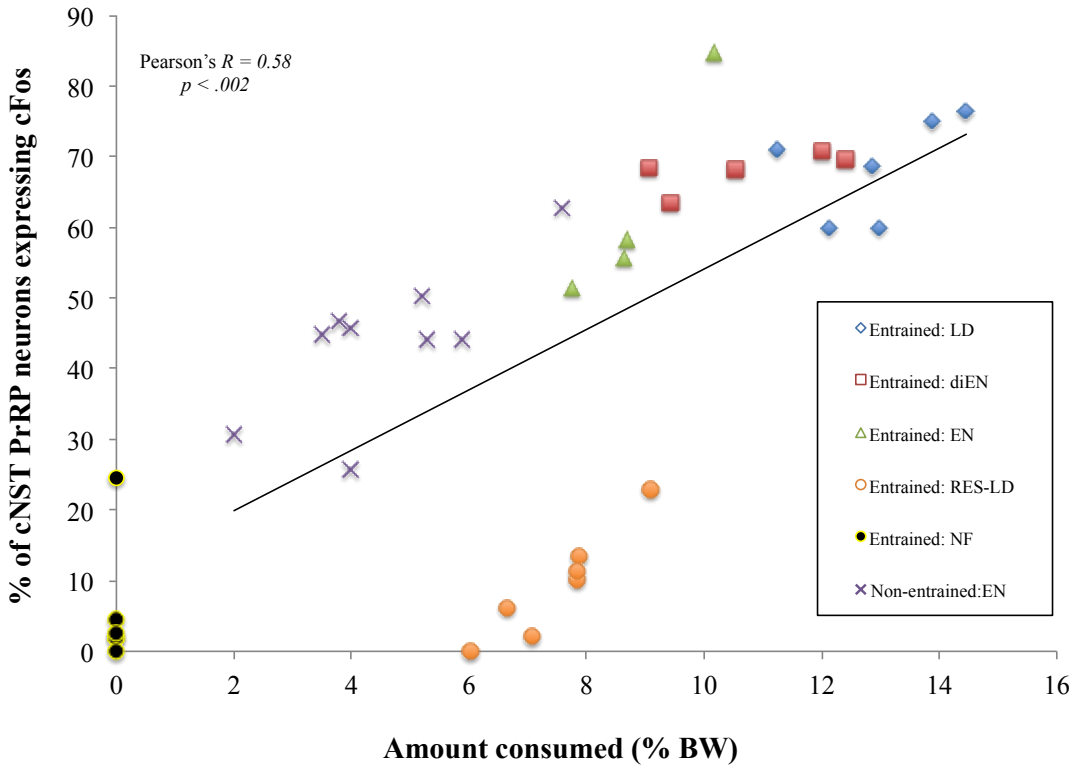

Figure 17. Meal size and activation of PrRP-positive neurons in meal-entrained rats

Relationship between amount consumed (as %BW) and cNTS PrRP neuronal activation in meal-entrained rats consuming different diets (Chapter 4, Experiment 1). Each symbol represents one rat. Data from non-entrained, Ensure-fed rats ("Non-entrained: EN") are included for comparison (as done in Figure 12). Data from Entrained: NF rats also are plotted for comparison, but the indicated correlation value is derived only from the five fed groups.

APPENDIX B

SATIATION AND STRESS-INDUCED HYPOPHAGIA: EXAMINING THE ROLE OF HINDBRAIN NEURONS EXPRESSING PROLACTIN-RELEASING PEPTIDE (PRRP) OR GLUCAGON-LIKE PEPTIDE 1 (GLP-1)

ABSTRACT

Neural circuits distributed within the brainstem, hypothalamus, and limbic forebrain interact to control food intake and energy balance under normal day-to-day conditions, and in response to stressful conditions under which homeostasis is threatened. Experimental studies using rats and mice have generated a voluminous literature regarding the functional organization of circuits that inhibit food intake in response to satiety signals, and in response to stress. Although the central neural bases of satiation and stress-induced hypophagia often are studied and discussed as if they were distinct, we propose that both behavioral states are generated, at least in part, by recruitment of two separate but intermingled groups of caudal hindbrain neurons. One group comprises a subpopulation of noradrenergic (NA) neurons within the caudal nucleus of the solitary tract (cNTS; A2 cell group) that is immunopositive for prolactin-releasing peptide (PrRP). The second group comprises non-adrenergic neurons within the cNTS and nearby reticular formation that synthesize glucagon-like peptide 1 (GLP-1). Axonal projections from

PrRP and GLP-1 neurons target distributed brainstem and forebrain regions that shape behavioral, autonomic, and endocrine responses to actual or anticipated homeostatic challenge, including the challenge of food intake. Evidence reviewed in this article supports the view that hindbrain PrRP and GLP-1 neurons contribute importantly to satiation and stress-induced hypophagia by modulating the activity of caudal brainstem circuits that control food intake. Hindbrain PrRP and GLP-1 neurons also engage hypothalamic and limbic forebrain networks that drive parallel behavioral and endocrine functions related to food intake and homeostatic challenge, and modulate conditioned and motivational aspects of food intake.

INTRODUCTION

Factors that increase or decrease food intake do so by altering meal size, meal frequency, or both (Smith, 1998; Smith, 2000). Satiation – the natural process that ends a meal – is a brainstem-mediated phenomenon in which food intake is terminated as a consequence of intake within that meal, thus influencing meal size. In contrast, satiety is a post-ingestive state that precludes initiation of a meal, thereby influencing meal frequency. Satiation occurs in adult decerebrate rats in which the brainstem is surgically isolated from the hypothalamus and the rest of the forebrain (Grill and Norgren, 1978a; Seeley et al., 1994; Grill and Kaplan, 2002; Grill, 2010), and in neonatal rats with functionally immature forebrain-brainstem connections (Rinaman et al., 1994). Satiation depends on peripherally-generated "satiety signals", which decrease activity in brainstem circuits that maintain ingestive licking/chewing/swallowing behaviors, and/or increase activity in brainstem circuits that suppress these behaviors. Although the brainstem is sufficient for satiation, the amount of food consumed before a meal is voluntarily terminated is powerfully modulated by neural signals from the forebrain and hormonal factors that increase or decrease

the behavioral potency of satiety signals (Grill and Hayes, 2012). Some of these signals and factors act directly on the brainstem components of ingestive control circuits, while others act indirectly by engaging hypothalamic and limbic forebrain regions that influence the activity of brainstem ingestive control circuits via descending projections (Smith, 1998; Smith, 2000; Grill and Kaplan, 2002; Luckman and Lawrence, 2003; Smith, 2004; Grill and Hayes, 2009; Grill, 2010). Satiety signals and other feedback about the quality and quantity of food consumed are delivered to brainstem and forebrain regions that coordinate a host of feeding-related processes, including anticipatory and reflexive metabolic, endocrine, and autonomic adjustments, preference and avoidance learning, appetitive motivation, and behavioral state control (Grill and Kaplan, 2002; Luckman and Lawrence, 2003; Smith, 2004; Grill and Hayes, 2009; Grill, 2010; Rinaman, 2010; Berthoud et al., 2011; Grill and Hayes, 2012).

Satiation and satiety are normal, everyday processes. However, consuming a satiating meal can be stressful, especially if food intake occurs at an unusual (i.e., unpredicted) time, or if the meal is unusually large or calorically dense. Food intake presents an immediate physiological challenge to homeostasis (Woods, 1991), and there is evidence that feeding activates the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis to increase circulating levels of glucocorticoids (i.e., cortisol in humans, corticosterone in rats and mice) (Shiraishi et al., 1984; Dallman et al., 2004). The HPA axis is primarily involved in energy storage and mobilization under baseline conditions and in response to homeostatic challenge (Dallman et al., 2004), and stressors are commonly defined as internal or external stimuli that increase HPA axis activity above baseline circadian-modulated levels. Meals are fundamentally disruptive to homeostasis because they cause significant changes in a variety of important physiological parameters that are under constant surveillance and regulation, such as gastrointestinal

distension, liver temperature, osmotic pressure, and blood glucose (Woods, 1991; Woods and Ramsay, 2000). Viewed from this perspective, it is reasonable to propose that there is a very fine line between central circuits that inhibit food intake during satiation and satiety, and those that inhibit intake during acute stress. The idea that satiety signals and hypophagic stressors might recruit a common set of neurons and circuits is not new [e.g., see (Ritter et al., 1999; Seeley et al., 2000; Calvez et al., 2011)], but the putatively shared circuits whose recruitment results in decreased meal size and/or frequency remain to be identified.

The present review discusses evidence that inhibition of food intake by satiety signals and by hypophagic stressors is mediated, at least in part, by recruitment of two phenotypically distinct but anatomically intermingled populations of hindbrain neurons. The first population comprises noradrenergic (NA) neurons within the caudal nucleus of the solitary tract (cNTS; A2 cell group), a majority of which express prolactin-releasing peptide (PrRP; Maruyama et al., 2001). PrRP was identified as an endogenous ligand for the human orphan G-protein-coupled receptor hGR3/GPR10, and earned its name because it induces prolactin secretion from anterior pituitary cells *in vitro* (Hinuma et al., 1998). However, PrRP is absent from the external layer of the median eminence, and there is no evidence that endogenous PrRP plays any physiological role in prolactin release. Instead, mRNA for PrRP receptor (hGR3/GPR10) is expressed in multiple brainstem and forebrain regions implicated in feeding, behavioral, and physiological responses to stress (Roland et al., 1999; Lawrence et al., 2000; Yamada et al., 2009). PrRP mRNA is expressed exclusively by a subset of caudal medullary NA neurons, and by a small number of neurons in a ventral region of the caudal dorsomedial hypothalamic nucleus (Iijima et al., 1999; Roland et al., 1999; Onaka et al., 2010). The second group of hindbrain neurons with a proposed role in both satiation and stress-induced hypophagia synthesize glucagon-like peptide 1

(GLP-1). Despite the largely overlapping hindbrain distribution of PrRP and GLP-1 neurons, the latter are a completely distinct population of non-adrenergic neurons that expresses mRNA for preproglucagon (PPG), the protein precursor of GLP-1. Within the brain, PPG mRNA expression is limited to the olfactory bulb, the cNTS, and the caudal medullary reticular formation (Larsen et al., 1997; Merchenthaler et al., 1999)[1]. Since PPG-expressing neurons within the olfactory bulb are interneurons with very short axons, GLP-1 fibers and terminals throughout the rest of the CNS can be assumed to originate from hindbrain PPG-expressing neurons.

Results from many published reports indicate that food intake in rats and mice is reduced after central infusions of PrRP, GLP-1, or their synthetic analogs (Tang-Christensen et al., 1996; Turton et al., 1996; Imeryuz et al., 1997; McMahon and Wellman, 1997; Asarian et al., 1998; McMahon and Wellman, 1998; Thiele et al., 1998; Lawrence et al., 2000; Kinzig et al., 2002; Lawrence et al., 2002; Schick et al., 2003b; Grabauskas et al., 2004; Lawrence et al., 2004; Bechtold and Luckman, 2006; Nakade et al., 2006b; Takayanagi et al., 2008; Holmes et al., 2009; Takayanagi and Onaka, 2010; Hayes et al., 2011b; Alhadeff et al., 2012). Such studies are important, and provide a strong foundation for the hypothesis that both neural populations drive hypophagia. However, delivery of synthetic peptides or their analogs into the brain is a poor model for understanding whether stimulus-induced release of endogenous PrRP or GLP-1 contributes to satiation or stress-induced hypophagia. The present review focuses on results from a smaller number of studies providing evidence that satiety signals and acute stress inhibit

[1] Amoeboid microglia also contain GLP-1 immunoreactive material, at least when activated in culture (Iwai T, Ito S, Tanimitsu K, Udagawa S, Oka J (2006) Glucagon-like peptide-1 inhibits LPS-induced IL-1beta production in cultured rat astrocytes. Neuroscience research 55:352-360.) suggesting that under certain pathological conditions activated microglia may release GLP-1 to access receptors in brain sites not normally targeted by the axons of hindbrain GLP-1 neurons.

food intake by recruiting endogenous PrRP and GLP-1 signaling pathways. Before reviewing those data, we first review the anatomical location, neurochemical features, and circuit connections of hindbrain PrRP and GLP-1 neurons.

ANATOMY OF THE DORSAL VAGAL COMPLEX AND ITS RESIDENT PRRP AND GLP-1 NEURONS

PrRP-immunopositive neurons and non-adrenergic GLP-1-immunopositive neurons are co-distributed in the hindbrain near the medullary-spinal junction, within caudal levels of the NTS and the nearby medullary reticular formation (Figure 18). The cNTS is the "visceral" NTS, distinct from the more rostral "gustatory" NTS (Lundy-Jr. and Norgren, 2004). The cNTS is a key component of the dorsal vagal complex (DVC), which also includes the area postrema (AP) and dorsal motor nucleus of the vagus (DMV). The DVC is remarkable for being perhaps the smallest circumscribed brain region whose destruction is incompatible with life. It is a critical central node for autonomic and endocrine functions, relaying interoceptive visceral, hormonal, and somatic feedback from body to brain, tuning stress responsiveness, and regulating glucose homeostasis and other aspects of energy balance (Zagon et al., 1999; Rinaman, 2003; Berthoud et al., 2006; Rinaman, 2007; Grill and Hayes, 2009; Grill, 2010; Rinaman, 2010; Zhang et al., 2010; Rinaman, 2011; Grill and Hayes, 2012). The AP and a significant portion of the subjacent cNTS contain fenestrated capillaries, allowing blood-borne factors to affect neurons in this region (Yamamoto et al., 2003). As recently reviewed (Grill and Hayes, 2009; Grill, 2010; Rinaman, 2010, 2011), AP neurons innervate the subjacent cNTS (Shapiro and Miselis, 1985; Kachidian and Pickel, 1993; Cunningham-Jr. et al., 1994), and cNTS neurons innervate other NTS neurons (including those located in the more rostral "taste" area) as well as gastrointestinal

and pancreatic vagal preganglionic parasympathetic motor neurons whose cell bodies occupy the DMV and whose dendrites ramify widely within the overlying cNTS (Shapiro and Miselis, 1985).

In addition to inputs from the AP, cNTS neurons receive sensory feedback from cardiovascular, respiratory, and alimentary systems (Kalia and Sullivan, 1982). Visceral sensory inputs arrive predominantly via glutamatergic glossopharyngeal and vagal afferents whose central axons converge in the solitary tract before synapsing with the dendrites and somata of cNTS neurons, including GLP-1 and NA neurons, the latter of which undoubtedly includes the PrRP-positive majority subpopulation (Altschuler et al., 1989a; Rinaman et al., 1989; Spyer, 1990; Bailey et al., 2006; Appleyard et al., 2007; Hisadome et al., 2010, 2011). In the mouse *in vitro* slice preparation, glutamatergic visceral afferent signals produce tightly synced, large-amplitude excitatory postsynaptic currents in NA and GLP-1 neurons within the cNTS, providing high-fidelity transmission of sensory nerve activity. Other visceral and somatic sensory inputs are relayed to the cNTS from the spinal cord, trigeminal and related nuclei, and reticular formation (Menétrey, 1987; Arbab et al., 1988; Altschuler et al., 1989a; Menétrey and dePommery, 1991; deSousaBuck et al., 2001).

Given the diversity of sensory signals they are positioned to receive, it is not surprising that GLP-1 and NA neurons, including PrRP neurons, respond to a broad array of interoceptive signals that can suppress food intake and also drive the HPA axis, including hormonal, thermal, osmotic, gastrointestinal, cardiovascular, respiratory, and inflammatory signals (Sawchenko and Swanson, 1981; Luckman, 1992; Rinaman and Levitt, 1993; Chan and Sawchenko, 1994; Rinaman et al., 1997; Callahan and Rinaman, 1998; Chan and Sawchenko, 1998; Dayas et al., 2001; Hollis et al., 2004; Rinaman, 2004; Myers et al., 2005; Myers and Rinaman, 2005; Duale

et al., 2007; Gaykema et al., 2007; Bienkowski and Rinaman, 2008; Kasparov and Teschemacher, 2008; Takayanagi et al., 2008; Bonnet et al., 2009; Rinaman, 2010). In these cited studies and many others, stimulus-induced "activation" of NA, PrRP, and/or GLP-1 neurons generally is characterized by double immunolabeling to localize nuclear cFos, the protein product of the immediate-early response gene, *c-fos*, together with cytoplasmic proteins identifying the chemical phenotype of activated neurons. As a binary index of neural activation, the presence or absence of cFos immunolabeling does not index the magnitude or duration of a neuron's presynaptic inputs, or its resulting spike frequency. However, quantitative and regional analyses of neural cFos expression permit assessment of stimulus-induced activation across multiple populations of phenotypically-identified neurons under control and experimental conditions, making it an ideal approach for testing hypotheses regarding neural sensitivity and/or function. The collective results of studies utilizing cFos indicate that NA, PrRP, and GLP-1 neurons are consistently activated by stimuli that present actual or anticipated threats to bodily homeostasis (see below, *Potential Role for PrRP and GLP-1 Neurons in Stress-Induced Hypophagia*).

PrRP and GLP-1 neurons participate in reciprocal connections with the medullary reticular formation, including the caudal ventrolateral medulla (cVLM), and other regions of the pons, diencephalon, and telencephalon that are implicated in food intake and body energy balance (Rinaman, 2010; Grill and Hayes, 2012). Direct descending projections from the cortex, limbic forebrain, and hypothalamus to cNTS regions where PrRP and GLP-1 neurons reside provide a route through which emotional and cognitive events can modulate visceral and ingestive responses to diverse threats and opportunities to which the organism is exposed, including conditioned responses that are based on past experience (Sawchenko, 1983; Li et al.,

1996; Li and Sawchenko, 1998; Woods and Ramsay, 2000; Dayas et al., 2001; Taché et al., 2001; Buller et al., 2003b; Dayas et al., 2004; Price, 2005; Blevins and Baskin, 2010). In turn, ascending projections from GLP-1 and NA neurons, including PrRP neurons, provide a route through which interoceptive feedback from the gastrointestinal tract and other organ systems can shape hypothalamic and limbic forebrain functions (Sawchenko, 1983; Loewy, 1990; Altschuler et al.; Onaka et al., 1995; Blessing, 1997; Rinaman, 2004; Onaka et al., 2010).

Beyond the cNTS: Many reports cited in the present review leave open the possibility that functions ascribed to central signaling by PrRP and/or GLP-1 neurons include signaling from neurons located not within the cNTS, but within the nearby medullary reticular formation. PrRP-positive neurons comprise a subset of the cVLM A1 noradrenergic cell group (Chen et al., 1999), whereas GLP-1 neurons are scattered in regions somewhat dorsal and medial to the A1 cell group (Vrang et al., 2007; Vrang and Grove, 2011). However, limited evidence suggests that PrRP and GLP-1 neurons within the cNTS are functionally distinct from those located within the reticular formation. For example, NA and GLP-1 neurons within the cNTS receive direct visceral sensory input (Hisadome et al., 2011), whereas those in the medullary reticular formation do not. This may explain why A2 NA neurons within the cNTS are recruited to express cFos in meal-entrained rats that consume a large scheduled meal, whereas cVLM A1 neurons are not activated (Rinaman et al., 1998). PrRP neurons within the cNTS also are activated in mice after a single cycle of 24-hr food deprivation followed by re-feeding, whereas PrRP neurons in the reticular formation are not (Takayanagi et al., 2008). In addition, the ability of hypoglycemia to increase food intake apparently is mediated by NA neurons within the VLM, and not by neurons within the cNTS A2 cell group (Li et al., 2009). There is no published

evidence that GLP-1 neurons within the cNTS vs. reticular formation project to different brain areas or maintain separate functions, although this possibility should be examined. It's relevant to note here that non-NA projections from the cNTS to the cVLM (Hermes et al., 2006) allow visceral signals to recruit neurons of the A1 cell group (Tucker et al., 1987; Yamashita et al., 1989; Kawano and Masuko, 1996; Bailey et al., 2006; Hermes et al., 2006), and the axons of many A1 neurons (including PrRP-positive neurons) join the ventral noradrenergic ascending bundle along with the axons of cNTS neurons that project rostrally from the hindbrain (Sawchenko and Swanson, 1981, 1982; Chan et al., 1995). In the absence of specific evidence to discriminate between PrRP or GLP-1 neurons within the cNTS vs. medullary reticular formation, a conservative approach dictates that projections and functions ascribed to chemically distinct neurons in either region should be considered likely to be shared by neurons in the other region.

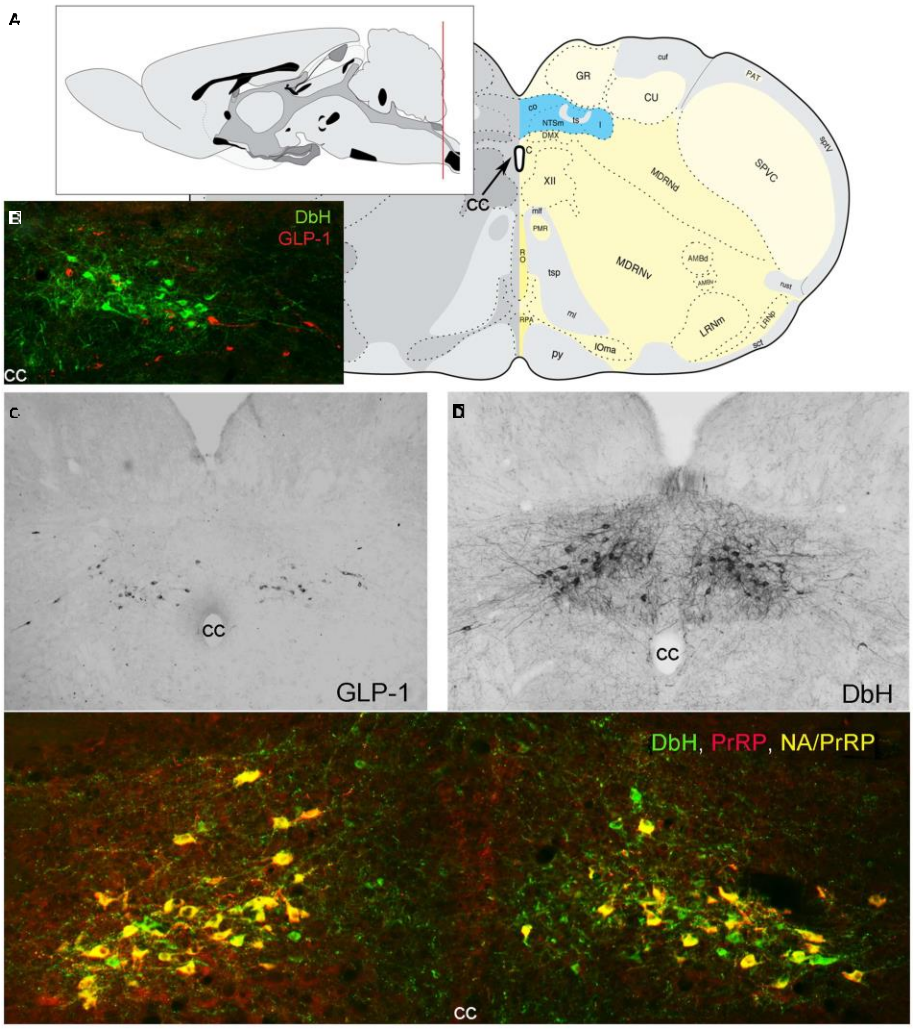

Figure 18. Location of PrRP-positive and GLP-1-positive neurons in the rat hindbrain.

A, Schematics illustrating the location of the cNTS (highlighted in blue), adapted from Swanson (2004). The red line in the mid-sagittal brain schematic at upper left illustrates the rostrocaudal level of all coronal sections depicted in Figure 18 images. **B,** In this image, dopamine beta hydroxylase (DbH) immunopositive NA neurons are green, while GLP-1-immunopositive neurons are red. The two intermingled populations are distinct, with no colocalization of

immunolabeling. **C,** GLP-1 immunoperoxidase-labeled neurons. **D,** DbH immunoperoxidase-positive NA neurons of the A2 cell group. **E,** In this image, all PrRP-positive neurons are double-labeled for DbH, rendering them yellow/orange (NA/PrRP neurons). Some intermingled NA neurons (green) are PrRP-negative. *cc, central canal.*

Other neurochemical features of PrRP and GLP-1 neurons: PrRP neurons are phenotypically distinguished by mRNA expression and positive immunolabeling for PrRP as well as tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, together with dopamine beta hydroxylase (DbH), the enzyme that converts dopamine to norepinephrine (NE) (Armstrong et al., 1982) (see Figure 18 for colocalization of PrRP and DbH immunolabeling within the cNTS). A1 and A2 neurons do not express phenylethanolamine N-methyltransferase, the enzyme that converts NE to epinephrine and identifies adrenergic neurons of the C1, C2, and C3 cell groups (Dahlström and Fuxe, 1964), which do not express PrRP (Morales et al., 2000). When considering the functional role of PrRP neurons and their axonal projections, it's important to keep in mind that these neurons release additional signaling molecules from their axon terminals and varicosities. In rats, at least 80% of A2 neurons express mRNA for a homolog of the vesicular glutamate transporter-2 (Stornetta et al., 2002), suggesting that the majority (perhaps all) of PrRP neurons release glutamate along with NE and PrRP from their axon terminals. In addition, subpopulations of catecholaminergic NTS neurons are immunopositive for neuropeptide Y (Sawchenko et al., 1985; Everitt and Hökfelt, 1989), nesfatin-1 (Bonnet et al., 2009), dynorphin (Ceccatelli et al., 1992), neurotensin (Riche et al., 1990), and/or pituitary adenylate cyclase-activating polypeptide (Das et al., 2007). The extent to which cNTS PrRP neurons co-express these additional signaling molecules remains unclear.

After posttranslational processing by the prohormone convertases PC1/3 and PC2, PPG-expressing neurons generate GLP-1 and several additional peptides for which GLP-1 neurons are immunopositive, including GLP-2, glicentin, intervening peptide-2, and oxyntomodulin (Schafer et al., 1993; Baggio and Drucker, 2007; Vrang and Larsen, 2010). Indirect evidence suggests that beyond PPG-encoded peptides, GLP-1 neurons also are immunopositive for met-enkephalin,

somatostatin, and inhibin-β (Sawchenko, 1988; Sawchenko et al., 1990; Sawchenko and Pfeiffer, 1995). Apparently, none of these signaling molecules are expressed by cNTS PrRP neurons (because none are expressed by NA neurons), and none of the neuropeptides that potentially are co-expressed by PrRP neurons (see previous section) have been localized to GLP-1 neurons. Another notable difference exists between PrRP and GLP-1 neurons in their expression of leptin receptors (Hay-Schmidt et al., 2001). Evidence for direct neuronal sensitivity to leptin has only been presented by one study, in which leptin directly depolarized identified GLP-1 neurons in brainstem slice preparations from transgenic mice (Hisadome et al., 2010). In mice, leptin receptor mRNA is expressed by GLP-1 neurons but not by NA or PrRP-positive cNTS neurons (Garfield et al., 2012). Conversely, in rats, NA (and PrRP) neurons express leptin receptor immunolabeling (Ellacott et al., 2002) and exhibit pSTAT3 induction after ip leptin administration (Huo et al., 2008), evidence for direct leptin sensitivity. Rat GLP-1 neurons do not exhibit pSTAT3 induction after ip leptin (Huo et al., 2008), but it is not known whether rat GLP-1 neurons express leptin receptors. Thus, not only do PrRP and GLP-1 neurons appear to display differential leptin sensitivity, their sensitivity appears to be reversed between rats and mice.

Brainstem and forebrain targets of PrRP and GLP-1 neurons: PrRP and GLP-1 neurons are well-positioned to participate in vago-vagal reflexes that modulate gastrointestinal motility, pancreatic hormone release, and other digestive-related autonomic processes associated with satiation and stress-induced hypophagia. GLP-1 and NA neurons, including PrRP neurons, project locally within the DVC and medullary reticular formation, and also to the spinal cord, comprising a subset of pre-autonomic hindbrain neurons implicated in autonomic control of

cardiovascular and digestive functions (Fukuda et al., 1987; Rogers et al., 2003; Martinez-Peña-y-Valenzuela et al., 2004; Hermann et al., 2005; Travagli et al., 2006; Duale et al., 2007; Pearson et al., 2007; Llewellyn-Smith et al., 2011; Llewellyn-Smith et al., 2013). Pancreatic and gastric vagal motor neurons express GLP-1R in rats (Wan et al., 2007; Holmes et al., 2009), and the ability of restraint stress to impact intestinal motility is blocked by central GLP-1R antagonism (Gulpinar et al., 2000). Intra-DVC or 4th ventricular microinjection of PrRP or GLP-1 has pronounced effects on vagally-mediated gastric motility, and results from *in vitro* slice preparations suggest that PrRP regulates gastric motor function by modulating the efficacy of excitatory synaptic inputs to vagal motor neurons (Grabauskas et al., 2004).

Axons and varicosities arising from PrRP and GLP-1 neurons also occupy regions of the spinal cord and pontine and medullary reticular formation that contain the pattern generators, pre-motor neurons, and motor neurons that control ingestive consummatory behaviors (i.e., licking/chewing/swallowing) (Norgren, 1978; Travers et al., 1997; Chen et al., 2001; Yano et al., 2001; Grill and Kaplan, 2002; Travers and Rinaman, 2002; Chen and Travers, 2003; Grill, 2010). Thus, PrRP and/or GLP-1 neurons may control the behavioral output of ingestive circuits to thereby induce or shape satiation and stress-induced hypophagia (Figure 19). Additional support comes from a transneuronal viral tracing study demonstrating that cNTS neurons provide synaptic input to oral pre-motor or motor neurons (Travers and Rinaman, 2002) with demonstrated importance for feeding control (Travers et al., 1997; Travers et al., 2010). It remains to be determined whether PrRP and GLP-1 neurons are among the cNTS neurons that are synaptically linked to ingestive pattern generators and oral motor output circuits.

Dual-labeling retrograde tracing and fiber lesion studies indicate that NA neurons, including PrRP-positive neurons, and GLP-1 neurons also project to multiple higher brain regions implicated in behavioral and physiological components of food intake (Morales et al., 2000; Renner et al., 2010; Rinaman, 2010, 2011; Renner et al., 2012). PrRP- and GLP-1-positive fibers have been localized to every medullary, pontine, mesencephalic, diencephalic, and limbic forebrain region that receives axonal input from the cNTS. Subsets of A2 neurons, presumably including PrRP neurons, have axon collaterals that innervate two or more forebrain targets (Petrov et al., 1993; Banihashemi and Rinaman, 2006; Schiltz and Sawchenko, 2007; Bienkowski and Rinaman, 2008). One study reported that 11-20% of hindbrain GLP-1 neurons were retrogradely labeled after either PVN or DMH tracer injections, but relatively few (i.e., 15-25%) of the tracer-labeled neurons projected to both hypothalamic nuclei (Vrang et al., 2007). On the other hand, 30-40% of all hindbrain GLP-1 neurons reportedly innervate the midbrain ventral tegmental area (VTA) or the ventral striatal nucleus accumbens (NAcc) (Alhadeff et al., 2012), indicating that subsets of GLP-1 neurons probably send collateralized axonal projections to multiple brain regions. Some individual A2 neurons have axons that collateralize to regions of the medullary reticular formation as well as to the limbic forebrain (Reyes and Bockstaele, 2006). Interestingly, however, individual A2 neurons appear to target either the pons or the VLM, but not both (Hermes et al., 2006), suggesting a higher degree of anatomical specificity for projections within the brainstem vs. projections to the hypothalamus and limbic forebrain.

Regarding hypothalamic projections, of particular relevance to the present review is evidence that PrRP and GLP-1 axonal projections target the medial parvocellular subregion of the paraventricular nucleus of the hypothalamus (mpPVN), where they form synaptic contacts with corticotropin releasing hormone (CRH)-positive neurons (Liposits et al., 1986; Matsumoto

et al., 2000; Sarkar et al., 2003) at the apex of the HPA axis. CRH is the principal and obligate hypophysiotropic peptide driving the HPA axis under basal conditions and in response to homeostatic challenge (Plotsky et al., 1989; Watts, 1996), and PrRP acts synergistically with NE to activate CRH neurons and the HPA axis (Maruyama et al., 2001; Seal et al., 2002; Uchida et al., 2010). Lesions that decrease NA input to the mpPVN markedly attenuate CRH neuronal cFos activation responses to interoceptive signals (Li et al., 1996; Fraley and Ritter, 2003; Rinaman, 2003; Ritter et al., 2003; Rinaman, 2007; Schiltz and Sawchenko, 2007; Bienkowski and Rinaman, 2008). Central administration of PrRP or GLP-1 activates cFos in the large majority of CRH-positive mpPVN neurons, and also increases plasma levels of corticosterone (Turton et al., 1996; Rowland et al., 1997; Kinzig et al., 2003; Mera et al., 2006). Although these cFos results by themselves do not prove that CRH neurons are activated directly by PrRP, NE, or GLP-1 receptor-mediated stimulation, bath application of GLP-1 to mouse hypothalamic slices increase spike frequency in a majority of PVN neurons (Acuna-Goycolea et al., 2004). Evidence for a mediating role of endogenously released GLP-1 comes from experiments demonstrating that the ability of stress to activate the HPA axis is markedly attenuated in rats after third ventricular administration of a GLP-1 receptor (GLP-1R) antagonist (Kinzig et al., 2003). Interestingly, however, GLP-1R -/- mice display paradoxically *increased* plasma corticosterone levels in responses to acute stress (MacLusky et al., 2000), suggesting that one role of endogenous GLP-1R signaling (at least in mice) may be to restrain or limit stress hormone secretion. This is consistent with the idea that GLP-1 signaling may serve a protective role to limit stress responses in the face of threat, e.g., to limit fever after immune challenge (Rinaman, 2000), and perhaps to guard against the overconsumption of unanticipated and/or excessively large meals.

As discussed in the Introduction (section 1.0), the HPA axis is activated by real or perceived homeostatic threats, including *ad libitum* (ad lib) or deprivation-induced food intake. By virtue of their synaptic inputs to CRH neurons, we hypothesize that PrRP and GLP-1 neurons may drive HPA axis responses to food intake, because PrRP and GLP-1 neurons drive HPA axis responses to some (but not all) experimental stressors (Rinaman, 2010, 2011).[2] In the mature, intact brain, PrRP and GLP-1 neurons innervate not only brainstem but also hypothalamic and limbic forebrain regions that control parallel autonomic, endocrine, and behavioral aspects of satiation and stress-induced hypophagia. In this regard, we view HPA axis activation as occurring in parallel with, but relatively independent from, the brainstem-mediated behavioral processes of satiation and hypophagia, which do not depend on neural connections between the brainstem and forebrain (Grill and Norgren, 1978a; Hall and Bryan, 1980; Hall and Swithers-Mulvey, 1992; Rinaman et al., 1994; Seeley et al., 1994; Grill and Kaplan, 2002). The following sections review evidence that PrRP and GLP-1 signaling pathways participate in satiation, and the final section (*Potential Role of PrRP and GLP-1 Neurons in Stress-induced Hypophagia*) reviews evidence that similar signaling pathways contribute to stress-induced hypophagia.

[2] HPA axis recruitment by PrRP and GLP-1 signaling pathways is discussed further in section 4.0 of this review.

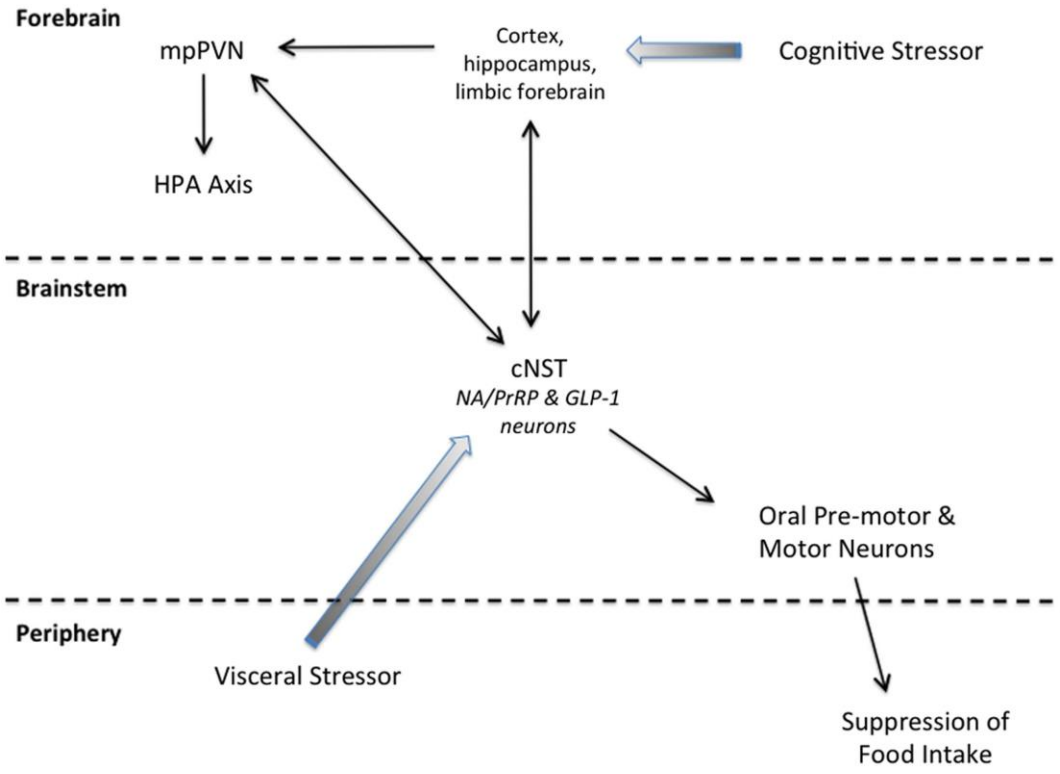

Figure 19. Summary schematic of our working hypothesis.

Cognitive stressors originate from conditioned and unconditioned cues that are processed through the cortex, hippocampus, and/or limbic forebrain before engaging the HPA axis and cNTS. Visceral stressors typically are unconditioned stimuli that first engage cNTS neurons (including PrRP and GLP-1 neurons) that innervate hypothalamic and limbic forebrain targets to recruit the HPA axis. Neurons within the cNTS engage brainstem targets to organize ingestive motor output.

POTENTIAL ROLE OF PRRP AND GLP-1 NEURONS IN SATIATION

To examine whether endogenous NA and/or GLP-1 signaling pathways contribute to normal feeding-induced satiation, a few studies have examined whether phenotypically identified NA or GLP-1 neurons within the cNTS, the former presumably including PrRP-positive neurons, are differentially activated to express cFos in rats that have recently consumed a large satiating meal, a smaller non-satiating meal, or no meal (Rinaman et al., 1998; Rinaman, 1999a; Gaykema et al., 2009; Kreisler and Rinaman, 2012). In other studies, pharmacological and genetic manipulations have been used to investigate whether rats or mice consume more food when the central receptor-mediated effects of endogenously released PrRP or GLP-1 are attenuated or eliminated [e.g., (Schick et al., 2003a; Takayanagi et al., 2008; Hayes et al., 2009; Barrera et al., 2011; Dossat et al., 2011; Alhadeff et al., 2012; Dickson et al., 2012)]. These general experimental approaches are among the best currently available for testing hypotheses about the endogenous central neural underpinnings of satiation and/or stress-induced hypophagia. However, the results of such studies must be interpreted within their own unique experimental context, with particular attention paid to the feeding paradigm utilized and any requisite surgical manipulations and handling.

Feeding-induced activation of PrRP and GLP-1 neurons: In the absence of experimental manipulations, the cNTS displays very low levels of neuronal cFos expression in ad lib-fed (presumably satiated) adult rats that are killed during the first few hours of the light cycle. Under such conditions, identified PrRP and NA neurons rarely express cFos in rats or mice (Shirohzu et al., 2008; Maniscalco et al., 2012a). Conversely, in alternate tissue sections from the same satiated rats, approximately 20% of identified GLP-1 neurons express cFos (Maniscalco et al.,

2012a). This moderate level of "baseline" GLP-1 activation is markedly reduced (i.e., from ~20% to less than 4%) if rats are not allowed to eat for 16-24 hours before sacrifice (Kreisler and Rinaman, 2012; Maniscalco et al., 2012a). Insofar as neuronal sensitivity is revealed by cFos labeling, these findings suggest that GLP-1 neurons are more sensitive than PrRP neurons to signals associated with post-prandial satiety in non-manipulated, ad lib-fed rats. Post-prandial satiety signals, which may be distinct from signals that promote satiation within a meal, could include gastrointestinal/colonic distension, post-absorptive nutrient levels, and circulating factors such as GLP-1, leptin, insulin, or ghrelin, for which plasma levels differ significantly in satiated rats and mice compared to levels measured after food deprivation (Mizuno et al., 1999; Kmiec et al., 2005; Kmiec et al., 2006; Johansson et al., 2008).

Under ad lib feeding conditions, laboratory rats typically maintain their body weight by consuming a large number (e.g., 10-15) of small meals each day, with ~80% of daily food intake occurring nocturnally, and the largest meals predictably consumed near the beginning and end of the dark phase of the photoperiod (Kissileff, 1970; Strubbe et al., 1986; Collier and Johnson, 1990; Woods, 2002). With few exceptions, published studies examining central cFos responses to feeding-induced signals use paradigms in which rats are acclimated over the course of several days or longer to a repeating schedule of food deprivation followed by re-feeding, in order to train them to voluntarily consume a meal that is unusually large compared to the typical size of an ad lib meal. Over the course of a week or two, through a Pavlovian process of classical conditioning, the animal learns to anticipate how much food can be safely consumed during the re-feeding period (including the approximate caloric and macronutrient composition of the meal) as it learns to initiate appropriately-timed cephalic phase responses (e.g., increased insulin release, gastric relaxation, digestive enzyme secretion) to ensure that larger meals can be safely

consumed (Woods and Ramsay, 2000; Woods, 2002). As pointed out in the Introduction (1.0), these anticipatory adjustments are vitally important, because the energetic benefits of eating are counterbalanced by the energetic cost of homeostatic challenge (Woods, 1991, 2002). Progressive meal-induced recruitment of visceral sensory "satiety" signals from the gastrointestinal tract to the hindbrain serve to constrain meal size during both ad lib and scheduled meal feeding, thereby limiting the stressful effects of food intake (Smith, 1998; Woods, 2002) through a process termed "meal tolerance" (Woods and Ramsay, 2000).

When experimentally naïve rats are food deprived for 24 hr and then re-fed for the very first time on chow or palatable liquid diet early in the dark cycle, both NA and GLP-1 neuronal populations are activated to express cFos in direct proportion to the gastric distension produced by the meal (Kreisler and Rinaman, 2012), suggesting that both PrRP and GLP-1 neurons might contribute to meal-induced satiation in this experimental context. Although the PrRP-positive subpopulation of NA neurons has not yet been examined for feeding-induced activation in rats, first-time re-feeding after a 24-hr fast does activate PrRP neurons in experimentally naïve mice (Takayanagi et al., 2008). After several days of acclimation to a repeating schedule of overnight food deprivation followed by a predictable solid or liquid morning meal, rats voluntarily consume an even larger amount, and cNTS NA neurons, presumably including PrRP neurons, still are acutely activated in proportion to the gastric distension produced by the meal (Rinaman et al., 1998). However, in the same rats, GLP-1 neurons are not activated (Rinaman, 1999a). Considered together, these findings suggest that NA and PrRP neurons contribute to meal-induced satiation in both experimental contexts, whereas GLP-1 neurons adapt or acclimate to signals such as gastric distension, elevated blood glucose, or insulin secretion that are predictably generated by a large scheduled meal. Indeed, the hypothesized acclimation of GLP-1 neurons

during scheduled meal feeding may be part of the Pavlovian process through which meal-entrained rats learn to tolerate the stress of consuming larger meals. In other words, a lack of GLP-1 neuronal recruitment may represent attenuation or removal of a "brake" on intake that would otherwise constrain meal size via engagement of GLP-1 receptors in the caudal brainstem, which reduces meal size (Hayes et al., 2008; Grill and Hayes, 2009; Hayes et al., 2009). If so, then GLP-1 neural recruitment by food intake in rats that are food-deprived and then re-fed for the very first time may help explain why these rats consume a smaller meal compared to acclimated, meal-entrained rats.

Increased food intake after pharmacological blockade of endogenous PrRP and GLP-1 signaling: There currently are no available pharmacological tools with which to antagonize PrRP (hGR3/GPR10) receptors. However, central administration of a monoclonal anti-PrRP antibody in rats was reported to increase meal size but not meal frequency, and to increase total food intake compared to the effects of a control antibody (Takayanagi et al., 2008). These results support the hypothesis that endogenous PrRP signaling participates in meal-induced satiation, but it is unclear where in the brain the proposed signaling occurs or whether satiation can be attributed to hindbrain populations of PrRP neurons as opposed to those located in the dorsomedial hypothalamus. It also is unclear whether meal size and total food intake measured in control rats after central injection of control antibody was reduced compared to similar measures in non-manipulated rats. The importance of including non-manipulated controls is discussed further, below (see the end of section 3.2).

Central GLP-1 signaling can be effectively disrupted by central administration of Exendin-9 (Ex-9), a specific GLP-1R antagonist. Daily intraventricular administration of Ex-9

produces daily increases in food intake compared to intake by rats after vehicle administration (Barrera et al., 2011), although it's not clear whether this effect depends on increased meal size (supporting a role in satiation), meal frequency (supporting a role in appetite/motivation), or both. Parenchymal administration of Ex-9 into subregions of the mesolimbic reward system, i.e., the VTA or NAcc, increases short-term intake of chow, palatable high fat diet, and sucrose in rats (Dossat et al., 2011; Alhadeff et al., 2012; Dickson et al., 2012), suggesting that GLP-1 signaling in these regions may normally act to suppress reward-driven intake. Ex-9 targeted to the lateral hypothalamus enhances short-term food intake in ad lib-fed rats, but has no effect on food intake in 24-hr food deprived rats (Schick et al., 2003b), perhaps because intake in deprived rats already is quite high. Conversely, Ex-9 injections targeted to the 4th ventricle or cNTS increase the amount of food consumed by rats after gastric distension, but not after intestinal nutrient infusions (Hayes et al., 2009). Results from these studies support the view that endogenous GLP-1 signaling suppresses or limits food intake across a variety of experimental conditions, and the cNTS/hindbrain may be an especially sensitive site of action for this effect (Grill and Hayes, 2012). Indeed, the hypothalamus and forebrain are not required for the ability of GLP-1 signaling to suppress gastric emptying and food intake in rats, as these responses are preserved in chronic supracollicular decerebrate rats (Hayes et al., 2008).

A potential interpretational problem in the studies cited above is the typical comparison of data from surgically-manipulated and/or drug-infused rats with data from control rats subjected to sham surgery and/or infused with vehicle. While these are appropriate experimental controls, they are incomplete. The manipulations employed in these studies are often complex, requiring one or more surgical sessions (e.g., to equip animals with chronic brain cannulas or intravenous catheters), and acute handling for central or systemic drug injection. Such

manipulations are themselves likely to promote some degree of stress-induced hypophagia, such that "baseline" food intake measured in animals after central or systemic vehicle treatment may be less than intake that would be observed under non-manipulated conditions. Accordingly, the ability of centrally administered Ex-9 or anti-PrRP antibody to increase food intake could be interpreted as evidence that central GLP-1 or PrRP signaling attenuates stress-induced hypophagia. Some experiments have attempted to address this issue by pre-exposing animals to experimental handling and drug infusion conditions in order to habituate them to the potentially stressful aspects of those conditions. However, results in "habituated" animals rarely are compared to results obtained in non-manipulated animals, making it unclear whether or how the habituation procedure affected results. It will be important for future studies to include additional comparative data from non-manipulated controls.

Increased food intake after genetic manipulation of PrRP and GLP-1 signaling: To better understand the role of endogenous GLP-1 signaling in satiation and long-term energy balance, one research group used a knockdown strategy in which short hairpin RNA was microinjected into the cNTS of adult rats to suppress endogenous PPG expression; this produced a significant and long-lasting increase in daily food intake and body weight compared to control rats (Barrera et al., 2011). However, it might be argued that the brainstem surgery itself in that study had a marked and long-lasting effect to reduce food intake and body weight growth, and that knockdown of PPG expression merely attenuated the deleterious effects of surgery. Evidence challenging a physiological role for GLP-1 signaling in daily food intake control comes from research using GLP-1R -/- mice, which are lean and consume a similar number of daily calories compared to wild-type mice (Scrocchi et al., 1996). In considering this apparent

discrepancy in results, a recent review (Vrang and Larsen, 2010) pointed out that GLP-1R -/- mice display an apparent disturbance in satiation, such that termination of food intake is delayed early in the dark period, thereby prolonging the initial nocturnal meal. This is followed by a later suppression of intake to achieve caloric compensation [see Figure 5A in (Scrocchi et al., 2000)].

GPR10 (PrRP receptor)-deficient mice display hyperphagia under ad lib feeding conditions, but not in a one-time 16 hr fasting/re-feeding protocol (Gu et al., 2004). In addition, GPR10 is required for the ability of exogenously administered PrRP and CCK to inhibit food intake in mice (Bechtold and Luckman, 2006). PrRP-deficient mice also display hyperphagia and increased body weight when maintained either on normal chow or on a high-fat diet (Mochiduki et al., 2010). In another study, PrRP-deficient mice displayed increased meal size (but not frequency) under ad lib feeding conditions, increased intake after deprivation, and reduced responsiveness to the feeding-suppressive effects of exogenous cholecystokinin octapeptide (CCK) and leptin (Takayanagi et al., 2008), which endogenously function as satiety signals. Interestingly, a polymorphism in the GPR10 gene that abolishes binding of PrRP in brain slices does not affect the ability of exogenously administered PrRP to suppress food intake in rats (Ellacott et al., 2005), suggesting that the hypophagic effects of the endogenous peptide could also be mediated through another, as yet unidentified, receptor signaling mechanism in rats. Additional studies will be required to examine this issue. However, there is compelling evidence that a natural mutation of the GPR10 receptor (in addition to mutation of CCK-1 receptors) in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat underlies its obese phenotype, and OLETF rats are insensitive to the hypophagic effects of exogenously administered PrRP (Watanabe et al., 2005).

POTENTIAL ROLE OF PRRP AND GLP-1 NEURONS IN STRESS-INDUCED HYPOPHAGIA

To the extent that it has been examined, GLP-1, PrRP, and NA neurons within the cNTS express cFos in every experimental situation in which food intake is acutely inhibited and the HPA axis is activated (Bouton and Bolles, 1980; Callahan and Rinaman, 1998; Rinaman et al., 1998; Rinaman, 1999a, 2003; Vrang et al., 2003; Zhu and Onaka, 2003; Onaka, 2004; Rinaman, 2004; Mera et al., 2006; Gaykema et al., 2007; Gaykema et al., 2008; Bonnet et al., 2009; Gaykema et al., 2009; Jelsing et al., 2009; Uchoa et al., 2009; Rinaman, 2010). As if to emphasize the close relationship between brainstem neural recruitment and endocrine responses to hypophagic stressors, systemically administered amylin reduces meal size but does not activate NA or GLP-1 neurons, and amylin does not activate the HPA axis (meaning it is not stressful) (Potes and Lutz, 2010). Acute stressors that activate cNTS NA and GLP-1 neurons, inhibit food intake, and activate the HPA axis also inhibit gastric emptying, likely via direct or indirect NA-, PrRP-, and GLP-1-mediated effects on autonomic outflow (Callahan and Rinaman, 1998; Hellstrom and Naslund, 2001; Rinaman, 2003; Rogers et al., 2003; Grabauskas et al., 2004; Rinaman, 2004; Nakade et al., 2006a; Balcita-Pedicino and Rinaman, 2007; Seto et al., 2008; Hayes et al., 2009).

States of threatened homeostasis are met by a complex but generally predictable repertoire of physiological and behavioral stress responses (Chrousos, 1998; Kyrou and Tsigos, 2009), including suppression of food intake (Dess and Vanderweele, 1994; Calvez et al., 2011). Stress responses are adaptive in the short term, because they shift the allocation of behavioral and physiological resources away from procuring and storing energy, and towards mobilizing energy and altering behavior to cope with the homeostatic threat. Experimental stressors are diverse in nature and magnitude, but can be categorized as either visceral (a.k.a.

interoceptive/physiological), or cognitive (a.k.a. neurogenic/psychological). Visceral stressors typically comprise unconditioned stimuli that present a direct challenge to physiological homeostasis, such as dehydration, toxemia, infection, or gastrointestinal stimulation. Their ability to activate the HPA axis largely depends on direct and relayed projections from spinal and hindbrain viscerosensory neurons to CRH neurons within the mpPVN, including projections from PrRP and GLP-1 neurons (Figure 19). Cognitive stressors originate from conditioned and unconditioned cues that are processed through the cortex and hippocampus before engaging the limbic forebrain and hypothalamus (Figure 19). Cognitive stressors in rats and mice include predator cues, open illuminated spaces, restraint/immobilization, and conditioned stimuli previously associated with an interoceptive or cognitive stressor. Thus, cognitive stressors predict an impending challenge to homeostasis, including the challenge of a large meal, which can be a visceral stressor. At least some cognitive stressors (i.e., mild footshock and restraint) do not require hindbrain inputs to the mpPVN in order to activate the HPA axis. However, GLP-1, PrRP, and NA neurons are activated by these and other cognitive stressors (Li et al., 1996; Morales and Sawchenko, 2003; Zhu and Onaka, 2003; Maniscalco et al., 2012a), likely due to recruitment of descending inputs to the cNTS that arise from stress-sensitive regions of the hypothalamus and limbic forebrain (Dayas et al., 2001; Buller et al., 2003a; Dayas et al., 2004; Blevins and Baskin, 2010) (Figure 19). Our working hypothesis is that, similar to satiation, stress-induced hypophagia depends on the recruitment of PrRP and GLP-1 neurons that participate in stressor-induced decreases in meal size (Morley et al., 1985). We propose that PrRP and GLP-1 neurons participate in satiation and stress-induced hypophagia regardless of whether these neurons are recruited directly via interoceptive/viscerosensory inputs to the cNTS, or indirectly via descending projections from the hypothalamus and limbic forebrain (Figure 19).

However, the potential role of PrRP or GLP-1 neurons in mediating stress-induced hypophagia has thus far been examined in only a small number of experimental models. The following paragraphs highlight these relatively limited findings, which cumulatively support the view that the ability of stressors to recruit PrRP and/or GLP-1 neurons contributes importantly to their ability to suppress food intake.

Cholecystokinin octapeptide: CCK was the first peptide hormone proposed to act as a physiological within-meal satiety signal (Gibbs et al., 1973). Endogenous CCK is released from the upper intestine by nutrient stimulation during and after a meal, binding to peripheral CCK receptors to thereby increase the activity of glutamatergic vagal sensory inputs to the cNTS (Bednar et al., 1994). Without challenging the role of endogenous CCK as a satiety factor, synthetic CCK also has been used as a pharmacological tool to activate central neural circuits that respond to gastric vagal stimulation. Such studies have demonstrated that systemic CCK dose-dependently decreases meal size (West et al., 1984) and elicits cFos activation of cNTS neurons (Zittel et al., 1999), including NA neurons (Maniscalco et al., 2012a) that presumably co-express PrRP . Moderate to high doses of CCK (i.e., 10-100 μg/kg BW) activate GLP-1 and NA neurons, including PrRP neurons (Luckman, 1992; Rinaman et al., 1993; Rinaman et al., 1995; Verbalis et al., 1995; Lawrence et al., 2002; Bechtold and Luckman, 2006; Babic et al., 2009) that project to the PVN (Rinaman et al., 1995) and activate CRH and oxytocin neurons (Verbalis et al., 1991). Systemic CCK at doses of ~1-3 μg/kg BW, which many researchers would argue are within the physiological range, are "stressful" in that they elevate plasma levels of adrenocorticotropic hormone (ACTH) in rats (Kamilaris et al., 1992); higher CCK doses produce larger HPA axis responses. CCK delivered at a lower dose (i.e., 0.5 μg/kg BW) does

not activate the HPA axis (Kamilaris et al., 1992). Although hypophagic effects of lower doses of CCK (i.e., ≤ 0.5μg/kg BW) have been reported using various systemic routes of administration and dietary conditions, to our knowledge there are no reports of parallel HPA axis activation under these conditions. As the hypothalamus and the entire forebrain are unnecessary for the ability of CCK to inhibit intake (Grill and Smith, 1988), it follows that HPA axis activation also is unnecessary for CCK-induced hypophagia. However, in the absence of evidence indicating otherwise, exogenous CCK-induced hypophagia appears to be accompanied by HPA axis activation, presumably because exogenous CCK activates hindbrain neurons that inhibit food intake and neurons that activate hypothalamic CRH neurons.

A2 neurons, including PrRP neurons, are necessary for the ability of a moderate to high dose of CCK to reduce meal size, and also are necessary for CCK-induced activation of neurons within the PVN (Rinaman, 2003). Interestingly, DbH -/- mice (which cannot convert dopamine to NA) show no deficiencies in the ability of CCK to reduce food intake (Cannon and Palmiter, 2003), suggesting that PrRP rather than NE is the principal mediator of CCK-induced hypophagia, at least in mice. Indeed, a later study confirmed that PrRP signaling is necessary for the ability of exogenous CCK to suppress food intake in mice (Bechtold and Luckman, 2006). CCK activates cFos expression by GLP-1 neurons (Maniscalco & Rinaman, 2012; Rinaman, 1999), but GLP-1 neuronal recruitment appears to be insufficient to support CCK hypophagia in rats with A2 neuronal loss, which would include loss of cNTS PrRP neurons (Rinaman, 2003). However, there are no published reports indicating whether GLP-1R signaling is necessary for CCK-induced hypophagia in either rats or mice.

Lithium Chloride (LiCl): Peripheral administration of the nauseogenic agent LiCl, an experimental model of toxemia, potently increases plasma corticosterone and inhibits food intake in rats (McCann et al., 1989). Unlike satiation, LiCl reduces food intake in rats by reducing feeding frequency, without reducing meal size (West et al., 1987). While LiCl treatment activates cFos within A2 and GLP-1 neurons in rats and mice (Rinaman, 1999a, b; Lachey et al., 2005; Rinaman and Dzmura, 2007), one report indicates that a hypophagic dose of LiCl in rats does not activate the PrRP-positive subpopulation of A2 neurons (Lawrence et al., 2002). This result suggests that LiCl suppresses food intake through non-PrRP signaling pathways, and that only stimuli related to normal satiation are sufficient to recruit PrRP neurons, as hypothesized previously (Luckman and Lawrence, 2003). Pharmacological blockade of central GLP-1 receptors or selective lesions that destroy A2 neurons, likely including PrRP neurons, attenuates (but does not abolish) the ability of LiCl to inhibit food intake in rats (Rinaman, 1999b; Seeley et al., 2000; Kinzig et al., 2002; Rinaman and Dzmura, 2007), and central GLP-1R antagonism blunts LiCl-induced activation of the HPA axis (Kinzig et al., 2003). Central GLP-1R antagonism also decreases LiCl-induced cFos in the rat cNTS (Thiele et al., 1998), evidence that GLP-1R signaling contributes to LiCl-induced recruitment of cNTS neurons. Conversely, although LiCl activates GLP-1 neurons in mice, neither GLP-1R antagonism in wild type mice nor the absence of GLP-1R signaling in GLP-1R -/- mice attenuates the hypophagic effects of LiCl (Lachey et al., 2005). These disparate findings in rats and mice suggest important species differences in the role of GLP-1 signaling in responses to toxemia and other nauseogenic treatments.

Immune challenge: Experimental models of infection, including systemic lipopolysaccharide (LPS; the major outer membrane component of gram-negative bacteria), promote the release of pro-inflammatory cytokines, elevate plasma levels of ACTH and corticosterone (Sapolsky et al., 1987; Hansen et al., 2000; Serrats and Sawchenko, 2006), and dose-dependently suppress food intake (Uehara et al., 1989; Kaneta and Kusnecov, 2005). Bacterial infections, LPS administration, and cytokines drive central stress responses via receptors located on vagal afferent terminals (Watkins et al., 1995; Fleshner et al., 1998; Goehler et al., 1999; Hosoi et al., 2005) and/or on endothelial and perivascular cells (Sawchenko et al., 2000; Zhang et al., 2003), and signaling through both routes engages cNTS neurons. LPS administered into the brain ventricles in rats reduces food intake primarily by reducing meal size (Plata-Salaman and Borkoski, 1993). Conversely, systemic administration of LPS reduces intake by reducing meal frequency (Langhans et al., 1990; Langhans et al., 1993), suggesting that central and peripheral routes of administration engage different feeding control circuits. Systemic LPS increases hindbrain PrRP gene expression (Mera et al., 2006), and activates A2 neurons, GLP-1 neurons, and PVN neurons to express cFos (Rinaman, 1999a). PVN and HPA axis activation in response to immune challenge is significantly attenuated in rats after unilateral transection of ascending projections from the cNTS to the PVN that interrupt both PrRP and GLP-1 signaling pathways (Li et al., 1996), or by selective neurochemical lesions of NA neurons (likely including PrRP neurons) that innervate the PVN (Bienkowski and Rinaman, 2008). Grill and colleagues (Grill et al., 2004) demonstrated that LPS-induced hypophagia is dependent on hindbrain, but not forebrain, GLP-1R signaling. However, hypophagic (and HPA axis) responses to LPS are intact in GPR10 -/- mice (Bechtold and Luckman, 2006), evidence that PrRP signaling is unnecessary for stress-induced hypophagia in this species. Taken together,

these studies suggest that the hypophagic and HPA axis responses to bacterial infection and proinflammatory cytokines depend in large part on the recruitment of NA and GLP-1 neurons, while the role of PrRP signaling has been challenged in mice, and not yet explored in rats.

Immobilization/restraint: One of the most commonly used models of cognitive stress is physical restraint, which activates the HPA axis and suppresses food intake (Rybkin et al., 1997; Kinzig et al., 2008; Seto et al., 2008; Calvez et al., 2011). A recent study investigated whether restraint and forced swim stress inhibited food intake in rats by reducing meal size, meal number, or both. Similar to satiation, restraint and forced swim stress both reduced food intake by reducing meal size and duration (Calvez et al., 2011), supporting the view that these stressors engage circuits that also are engaged by satiety signals. Restraint increases PrRP gene expression (Mera et al., 2006) and also activates cFos in NA neurons, including PrRP neurons (Dayas et al., 2001; Maruyama et al., 2001; Banihashemi et al., 2011), apparently via descending projections from the PVN (Dayas et al., 2001; Dayas et al., 2004). Recent findings in our laboratory indicate that restraint also activates GLP-1 neurons in rats (Maniscalco et al., 2012a). The ability of restraint to inhibit food intake is closely linked to its ability to inhibit vagally-mediated gastric emptying (Seto et al., 2008; Suzuki and Hibi, 2010). However, it currently is unknown whether the ability of restraint or any other cognitive stressor to decrease gastric emptying and food intake depends on central PrRP or GLP-1 receptor signaling.

CONCLUSION

Stress affects both food intake and energy balance, and food intake can itself be stressful (Woods, 1991). For the purpose of this review, we set out to gather and interpret experimental

evidence that satiety signals and stress engage a common set of neurons that contribute to the inhibition of food intake. Hindbrain PrRP and GLP-1 neurons satisfy many of the criteria that one might consider important for such a common set of neurons. Both neuronal populations are recruited to express cFos in animals exposed to satiety signals and many hypophagic stressors, and PrRP and GLP-1 signaling pathways impact body energy balance by reducing food intake and activating the HPA axis. Based on our review of the available literature, we propose that hindbrain PrRP and GLP-1 neurons represent important points of central integration in the control of energy intake and metabolism during feeding and in response to other acute homeostatic challenges. We do not argue that PrRP and GLP-1 neurons are the only important players in these coordinated processes. Instead, we present this evidence to establish a working hypothesis about the unique role played by these cNTS neurons within the anatomically broad and complex neural systems that regulate energy homeostasis on a day-to-day basis. Experimental predictions arising from this hypothesis will be challenged by ongoing and future work in our laboratory.

BIBLIOGRAPHY

Abbott CR, Monteiro M, Small CJ, Sajedi A, Smith KL, Parkinson JR, Ghatei MA, Bloom SR (2005) The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. Brain Res 1044:127-131.

Aicher SA, Kurucz OS, Reis DJ, Milner TA (1995) Nucleus tractus solitarius efferent terminals synapse on neurons in the caudal ventrolateral medulla that project to the rostral ventrolateral medulla. Brain Res 693:51-63.

Aja S, Schwartz GJ, Kuhar MJ, Moran TH (2001) Intracerebroventricular CART peptide reduces rat ingestive behavior and alters licking microstructure. Am J Physiol Regul Integr Comp Physiol 280:R1613-1619.

Alhadeff AL, Rupprecht LE, Hayes MR (2012) GLP-1 neurons in the nucleus of the solitary tract project directly to the ventral tegmental area and nucleus accumbens to control for food intake. Endocrinology 153:647-658.

Altschuler SM, Rinaman L, Miselis RR (1992) Viscerotopic representation of the alimentary tract in the dorsal and ventral vagal complex in the rat. In: Neuroanatomy and Physiology of Abdominal Vagal Afferents (Ritter S, Ritter RC, Barnes CD, eds), pp 21-54. Boca Raton: CRC Press.

Altschuler SM, Bao X, Bieger D, Hopkins DA, Miselis RR (1989a) Viscerotopic representation of the upper alimentary tract in the rat: sensory ganglia and nuclei of the solitary and spinal trigeminal tracts. J Comp Neurol 283:248-268.

Altschuler SM, Bao XM, Bieger D, Hopkins DA, Miselis RR (1989b) Viscerotopic representation of the upper alimentary tract in the rat: sensory ganglia and nuclei of the solitary and spinal trigeminal tracts. J Comp Neurol 283:248-268.

Appleyard SM, Marks D, Kobayashi K, Okano H, Low MJ, Andresen MC (2007) Visceral afferents directly activate catecholamine neurons in the solitary tract nucleus. J Neurosci 27:13292-13302.

Arbab MA, Delgado T, Wiklund L, Svendgaard NA (1988) Brain stem terminations of the trigeminal and upper spinal ganglia innervation of the cerebrovascular system: WGA-HRP transganglionic study. Journal of Cerebral Blood Flow and Metabolism 8:54-63.

Archer ZA, Brown YA, Rayner DV, Stubbs RJ, Mercer JG (2006) Effect of flavour of liquid Ensure diet supplement on energy intake in male SD rats. Physiol Behav 89:414-419.

Armstrong DM, Ross CA, Pickel VP, Joh TH, Reis DJ (1982) Distribution of dopamine, noadrenaline and adrenaline-containing cell bodies in the rat medulla oblongata: Demonstration by immunocytochemical localization of catecholamine biosynthetic enzymes. J Comp Neurol 211:173-187.

Asarian L, Corp ES, Hrupka B, Geary N (1998) Intracerebroventricular glucagon-like peptide-1 (7-36) amide inhibits sham feeding in rats without eliciting satiety. Physiol Behav 64:367-372.

Babic T, Townsend RL, Patterson LM, Sutton GM, Zheng H, Berthoud H-R (2009) Phenotype of neurons in the nucleus of the solitary tract that express CCK-induced activation of the ERK signaling pathway. Am J Physiol Regul Integr Comp Physiol 296:R845-R854.

Baggio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. Gastroenterology 132:2131-2157.

Bailey TW, Hermes SM, Andresen MC, Aicher SA (2006) Cranial visceral afferent pathways through the nucleus of the solitary tract to caudal ventrolateral medulla or paraventricular hypothalamus: target-specific synaptic reliability and convergence patterns. J Neurosci 26:11893-11902.

Balcita-Pedicino JJ, Rinaman L (2007) Noradrenergic axon terminals contact gastric preautonomic neurons in the paraventricular nucleus of the hypothalamus in rats. J Comp Neurol 501:608-618.

Banihashemi L, Rinaman L (2006) Noradrenergic inputs to the bed nucleus of the stria terminalis and paraventricular nucleus of the hypothalamus underlie hypothalamic-pituitary-adrenal axis but not hypophagic or conditioned avoidance responses to systemic yohimbine. J Neurosci 26:11442-11453.

Banihashemi L, O'Neill EJ, Rinaman L (2011) Central neural responses to restraint stress are altered in rats with an early life history of repeated brief maternal separation. Neuroscience 192:413-428.

Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ (2011) Hyperphagia and Increased Fat Accumulation in Two Models of Chronic CNS Glucagon-Like Peptide-1 Loss of Function. J Neuroscience 31:3904-3913.

Bechtold DA, Luckman SM (2006) Prolactin-releasing Peptide mediates cholecystokinin-induced satiety in mice. Endocrinology 147:4723-4729.

Bednar I, Qian M, Qureshi GA, Kallstrom L, Johnson AE, Carrer H, Sodersten P (1994) Glutamate inhibits ingestive behaviour. J Neuroendocrinol 6:403-408.

Berthoud H-R, Sutton GM, Townsend RL, Patterson LM, Zheng H (2006) Brainstem mechanisms integrating gut-derived satiety signals and descending forebrain information in the control of meal size. Physiology and Behavior 89:517-524.

Berthoud HR, Lenard NR, Shin AC (2011) Food reward, hyperphagia, and obesity. Am J Physiol Regul Integr Comp Physiol 300:R1266-1277.

Bienkowski MS, Rinaman L (2008) Noradrenergic inputs to the paraventricular hypothalamus contribute to hypothalamic-pituitary-adrenal axis and central Fos activation in rats after acute systemic endotoxin exposure. Neuroscience 156:1093-1102.

Blessing WW (1997) The Lower Brainstem and Bodily Homeostasis. New York: Oxford University Press.

Blevins JE, Baskin DG (2010) Hypothalamic-brainstem circuits controlling eating. Forum of Nutrition 63:133-140.

Blundell J (2010) Making claims: functional foods for managing appetite and weight. Nature reviews Endocrinology 6:53-56.

Blundell JE, Bellisle F (2013) Satiation, satiety, and the control of food intake. Philadelphia: Woodhead Publishing Limited.

Bonnet MS, Pecchi E, Trouslard J, Jean A, Dallaporta M, Troadec J-D (2009) Central nesfatin-1-expressing neurons are sensitive to peripheral inflammatory stimulus. Journal of Neuroinflammation 6:27.

Bouton M, Bolles R (1980) Conditioned fear assessed by freezing and by the suppression of three different baselines. Animal Learning & Behavior 8:429-434.

Buller KM, Dayas CV, Day TA (2003a) Descending pathways from the paraventricular nucleus contribute to the recruitment of brainstem nuclei following a systemic immune challenge. Neuroscience 118:189-203.

Buller KM, Crane JW, Spencer SJ, Day TA (2003b) Systemic apomorphine alters HPA axis responses to interleukin-1b administration but not sound stress. Psychoneuroendocrinology 28:715-732.

Callahan JB, Rinaman L (1998) The postnatal emergence of dehydration anorexia in rats is temporally associated with the emergence of dehydration-induced inhibition of gastric emptying. Physiol Behav 64:683-687.

Calvez J, Fromentin G, Nadkarni N, Darcel N, Even P, Tome D, Ballet N, Chaumontet C (2011) Inhibition of food intake induced by acute stress in rats is due to satiation effects. Physiol Behav 104:675-683.

Cannon CM, Palmiter RD (2003) Peptides that regulate food intake: norepinephrine is not required for reduction of feeding induced by cholecystokinin. Am J Physiol Regul Integr Comp Physiol 284:R1384-1388.

Ceccatelli S, Seroogyb KB, Millhornc DE, Tereniusd L (1992) Presence of a dynorphin-like peptide in a restricted subpopulation of catecholaminergic neurons in rat nucleus tractus solitarii. Brain Res 589:225-230.

Chan RK, Sawchenko PE (1994) Spatially and temporally differentiated patterns of c-fos expression in brainstem catecholaminergic cell groups induced by cardiovascular challenges in the rat. J Comp Neurol 348:433-460.

Chan RKW, Sawchenko PE (1998) Organization and transmitter specificity of medullary neurons activated by sustained hypertension: implications for understanding baroreceptor reflex circuitry. J Neuroscience 18:371-387.

Chan RKW, Peto CA, Sawchenko PE (1995) A1 catecholamine cell group: fine structure and synaptic input from the nucleus of the solitary tract. J CompNeurol 351:62-80.

Chaudhuri A, Zangenehpour S, Rahbar-Dehgan F, Ye F (2000) Molecular maps of neural activity and quiescence. Acta neurobiologiae experimentalis 60:403-410.

Chen C, Dun SL, Dun NJ, Chang JK (1999) Prolactin-releasing peptide-immunoreactivity in A1 and A2 noradrenergic neurons of the rat medulla. Brain Res 822:276-279.

Chen Z, Travers JB (2003) Inactivation of amino acid receptors in medullary reticular formation modulates and suppresses ingestion and rejection responses in the awake rat. Am J Physiol Regul Integr Comp Physiol 285:R68-R83.

Chen Z, Travers SP, Travers JB (2001) Muscimol infusions in the brain stem reticular formation reversibly block ingestion in the awake rat. Am J Physiol Regul Integr Comp Physiol 280:R1085-R1094.

Chrousos GP (1998) Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. Ann N Y Acad Sci 851:311-335.

Collier G, Johnson DF (1990) The time window of feeding. Physiol Behav 48:771-777.

Cunningham-Jr. ET, Miselis RR, Sawchenko PE (1994) The relationship of efferent projections from the area postema to vagal motor and brain stem catecholamine-containing cell

groups: an axonal transport and immunohistochemical study in the rat. Neuroscience 58:635-648.
Dahlström A, Fuxe K (1964) Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol Scand 62, supplement 232:3-55.
Dallman MF, la Fleur SE, Pecoraro NC, Gomez F, Houshyar H, Akana SF (2004) Minireview: glucocorticoids--food intake, abdominal obesity, and wealthy nations in 2004. Endocrinology 145:2633-2638.
Das M, Vihlen CS, Legradi G (2007) Hypothalamic and brainstem sources of pituitary adenylate cyclase-activating polypeptide nerve fibers innervating the hypothalamic paraventricular nucleus in the rat. J Comp Neurol 500:761-776.
Davis JD (1989) The microstructure of ingestive behavior. Ann N Y Acad Sci 575:106-119; discussion 120-101.
Davis JD, Campbell CS (1973) Peripheral control of meal size in the rat. Effect of sham feeding on meal size and drinking rate. J Comp Physiol Psychol 83:379-387.
Dayas CV, Buller KM, Day TA (2004) Hypothalamic paraventricular nucleus neurons regulate medullary catecholamine cell responses to restraint stress. J Comp Neurol 478:22-34.
Dayas CV, Buller KM, Crane JW, Xu Y, Day TA (2001) Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. European Journal of Neuroscience 14:1143-1152.
de Lartigue G, de La Serre CB, Raybould HE (2011a) Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. Physiol Behav 105:100-105.
de Lartigue G, Barbier de la Serre C, Espero E, Lee J, Raybould HE (2011b) Diet-induced obesity leads to the development of leptin resistance in vagal afferent neurons. Am J Physiol Endocrinol Metab 301:E187-195.
de Lartigue G, Barbier de la Serre C, Espero E, Lee J, Raybould HE (2012) Leptin resistance in vagal afferent neurons inhibits cholecystokinin signaling and satiation in diet induced obese rats. PLoS One 7:e32967.
deSousaBuck H, Caous CA, Lindsey CJ (2001) Projections of the paratrigeminal nucleus to the ambiguus, rostroventrolateral and lateral reticular nuclei, and the solitary tract. Autonomic Neuroscience: Basic & Clinical 87:187-200.
Dess NK, Vanderweele DA (1994) Lithium chloride and inescapable, unsignaled tail shock differentially affect meal patterns of rats. Physiol Behav 56:203-207.
Dickson SL, Shirazi RH, Hansson C, Bergquist F, Nissbrandt H, Skibicka KP (2012) The Glucagon-Like Peptide 1 (GLP-1) Analogue, Exendin-4, Decreases the Rewarding Value of Food: A New Role for Mesolimbic GLP-1 Receptors. Journal of Neuroscience 32:4812-4820.
Dodd GT, Luckman SM (2013) Physiological Roles of GPR10 and PrRP Signaling. Frontiers in endocrinology 4:20.
Dossat AM, Lilly N, Kay K, Williams DL (2011) Glucagon-like peptide 1 receptors in nucleus accumbens affect food intake. J Neurosci 31:14453-14457.
Dossat AM, Diaz R, Gallo L, Panagos A, Kay K, Williams DL (2013) Nucleus accumbens GLP-1 receptors influence meal size and palatability. Am J Physiol Endocrinol Metab 304:E1314-1320.

Duale H, Waki H, Howorth P, Kasparov S, Teschemacher AG, Paton JFR (2007) Restraining influence of A2 neurons in chronic control of arterial pressure in spontaneously hypertensive rats. Cardiovasc Res 76:184-193.

Ellacott KL, Donald EL, Clarkson P, Morten J, Masters D, Brennand J, Luckman SM (2005) Characterization of a naturally-occurring polymorphism in the UHR-1 gene encoding the putative rat prolactin-releasing peptide receptor. Peptides 26:675-681.

Everitt BJ, Hökfelt T (1989) The coexistence of neuropeptide-Y with other peptides and amines in the central nervous system. In: Neuropeptide Y (Mutt V, Fuxe K, Hökfelt T, Lundberg JM, eds), pp 61-71. New York: Raven Press.

Fleshner M, Goehler LE, Schwartz BA, McGorry M, Martin D, Maier SF, Watkins LR (1998) Thermogenic and corticosterone responses to intravenous cytokines (IL-1b and TNF-a) are attenuated by subdiaphragmatic vagotomy. Journal of Neuroimmunology 86:134-141.

Fraley GS, Ritter S (2003) Immunolesion of norepinephrine and epinephrine afferents to medial hypothalamus alters basal and 2-deoxy-D-glucose-induced neuropeptide Y and agouti gene-related protein messenger ribonucleic acid expression in the arcuate nucleus. Endocrinology 144:75-83.

Fujii R, Fukusumi S, Hosoya M, Kawamata Y, Habata Y, Hinuma S, Sekiguchi M, Kitada C, Kurokawa T, Nishimura O, Onda H, Sumino Y, Fujino M (1999) Tissue distribution of prolactin-releasing peptide (PrRP) and its receptor. Regul Pept 83:1-10.

Fukuda A, Minami T, Nabekura J, Oomura Y (1987) The effects of noradrenaline on neurones in the rat dorsal motor nucleus of the vagus, in vitro. J Physiol 393:213-231.

Garfield AS, Patterson C, Skora S, Gribble FM, Reimann F, Evans ML, Myers MG, Jr., Heisler LK (2012) Neurochemical characterization of body weight-regulating leptin receptor neurons in the nucleus of the solitary tract. Endocrinology 153:4600-4607.

Gaykema RP, Park SM, McKibbin CR, Goehler LE (2008) Lipopolysaccharide suppresses activation of the tuberomammillary histaminergic system concomitant with behavior: a novel target of immune-sensory pathways. Neuroscience 152:273-287.

Gaykema RP, Daniels TE, Shapiro NJ, Thacker GC, Park SM, Goehler LE (2009) Immune challenge and satiety-related activation of both distinct and overlapping neuronal populations in the brainstem indicate parallel pathways for viscerosensory signaling. Brain Res 1294:61-79.

Gaykema RPA, Chen C-C, Goehler LE (2007) Organization of immune-responsive medullary projections to the bed nucleus of the stria terminalis, central amygdala, and paraventricular nucleus of the hypothalamus: evidence for parallel viscerosensory pathways in the rat brain. Brain Res 1130:130-145.

Gibbs J, Young RC, Smith GP (1973) Cholecystokinin decreases food intake in rats. J Comp Physiol Psychol 84:488-495.

Goehler LE, Gaykema RPA, Nguyen KT, Lee JE, Tilders FJH, Maier SF, Watkins LR (1999) Interleukin-1b in immune cells of the abdominal vagus nerve: a link between the immune and nervous systems? J Neuroscience 19:2799-2806.

Grabauskas G, Zhou SY, Das S, Lu Y, Owyang C, Moises HC (2004) Prolactin-releasing peptide affects gastric motor function in rat by modulating synaptic transmission in the dorsal vagal complex. J Physiol 561:821-839.

Grill HJ (2010) Leptin and the systems neuroscience of meal size control. Front Neuroendocrinol 31:61-78.

Grill HJ, Norgren R (1978a) Chronically decerebrate rats demonstrate satiation but not bait shyness. Science 201:267-269.
Grill HJ, Norgren R (1978b) The taste reactivity test. II. Mimetic responses to gustatory stimuli in chronic thalamic and chronic decerebrate rats. Brain Res 143:281-297.
Grill HJ, Smith GP (1988) Cholecystokinin decreases sucrose intake in chronic decerebrate rats. Am J Physiol 254:R853-856.
Grill HJ, Kaplan JM (1992) Sham feeding in intact and chronic decerebrate rats. Am J Physiol 262:R1070-1074.
Grill HJ, Kaplan JM (2002) The neuroanatomical axis for control of energy balance. Front Neuroendocrinol 23:2-40.
Grill HJ, Hayes MR (2009) The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. Int J Obes (Lond) 33 Suppl 1:S11-15.
Grill HJ, Hayes MR (2012) Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance. Cell Metab 16:296-309.
Grill HJ, Carmody JS, Amanda Sadacca L, Williams DL, Kaplan JM (2004) Attenuation of lipopolysaccharide anorexia by antagonism of caudal brain stem but not forebrain GLP-1-R. Am J Physiol Regul Integr Comp Physiol 287:R1190-1193.
Gu W, Geddes BJ, Zhang C, Foley KP, Stricker-Krongrad A (2004) The prolactin-releasing peptide receptor (GPR10) regulates body weight homeostasis in mice. Journal of Molecular Neuroscience : MN 22:93-103.
Gulpinar MA, Bozkurt A, Coskun T, Ulusoy NB, Yegen BC (2000) Glucagon-like peptide (GLP-1) is involved in the central modulation of fecal output in rats. Am J Physiol Gastrointest Liver Physiol 278:G924-929.
Hall WG, Bryan TE (1980) The ontogeny of feeding in rats. II. Independent ingestive behavior. J Comp Physiol Psychol 94:746-756.
Hall WG, Swithers-Mulvey SE (1992) Developmental strategies in the analysis of ingestive behavior. Ann N Y Acad Sci 662:1-15.
Hansen MK, Nguyen KT, Fleshner M, Goehler LE, Gaykema RP, Maier SF, Watkins LR (2000) Effects of vagotomy on serum endotoxin, cytokines, and corticosterone after intraperitoneal lipopolysaccharide. Am J Physiol Regul Integr Comp Physiol 278:R331-336.
Hay-Schmidt A, Helboe L, Larsen PJ (2001) Leptin receptor immunoreactivity is present in ascending serotonergic and catecholaminergic neurons of the rat. Neuroendocrinology 73:215-226.
Hayes MR, Skibicka KP, Grill HJ (2008) Caudal brainstem processing is sufficient for behavioral, sympathetic, and parasympathetic responses driven by peripheral and hindbrain glucagon-like-peptide-1 receptor stimulation. Endocrinology 149:4059-4068.
Hayes MR, Bradley L, Grill HJ (2009) Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling. Endocrinology 150:2654-2659.
Hayes MR, Kanoski SE, Alhadeff AL, Grill HJ (2011a) Comparative effects of the long-acting GLP-1 receptor ligands, liraglutide and exendin-4, on food intake and body weight suppression in rats. Obesity (Silver Spring) 19:1342-1349.
Hayes MR, Leichner TM, Zhao S, Lee GS, Chowansky A, Zimmer D, De Jonghe BC, Kanoski SE, Grill HJ, Bence KK (2011b) Intracellular signals mediating the food intake-

suppressive effects of hindbrain glucagon-like peptide-1 receptor activation. Cell Metab 13:320-330.
Hellstrom PM, Naslund E (2001) Interactions between gastric emptying and satiety, with special reference to glucagon-like peptide-1. Physiol Behav 74:735-741.
Hermann GE, Nasse JS, Rogers RC (2005) Alpha-1 adrenergic input to solitary nucleus neurones: calcium oscillations, excitation and gastric reflex control. J Physiol 562:553-568.
Hermes SM, Mitchell JL, Aicher SA (2006) Most neurons in the nucleus tractus solitarii do not send collateral projections to multiple autonomic targets in the rat brain. Exp Neurol 198:539-551.
Hinuma S, Habata Y, Fujii R, Kawamata Y, Hosoya M, Fukusumi S, Kitada C, Masuo Y, Asano T, Matsumoto H, Sekiguchi M, Kurokawa T, Nishimura O, Onda H, Fujino M (1998) A prolactin-releasing peptide in the brain. Nature 393:272-276.
Hisadome K, Reimann F, Gribble FM, Trapp S (2010) Leptin directly depolarizes preproglucagon neurons in the nucleus tractus solitarius: electrical properties of glucagon-like Peptide 1 neurons. Diabetes 59:1890-1898.
Hisadome K, Reimann F, Gribble FM, Trapp S (2011) CCK stimulation of GLP-1 neurons involves alpha1-adrenoceptor-mediated increase in glutamatergic synaptic inputs. Diabetes 60:2701-2709.
Hollis JH, Lightman SL, Lowry CA (2004) Integration of systematic and visceral sensory information by medullary catecholaminergic systems during peripheral inflammation. Ann N Y Acad Sci 1018:71-75.
Holmes GM, Browning KN, Tong M, Qualls-Creekmore E, Travagli RA (2009) Vagally mediated effects of glucagon-like peptide 1: in vitro and in vivo gastric actions. J Physiol 587:4749-4759.
Hosoi T, Okuma Y, Matsuda T, Nomura Y (2005) Novel pathway for LPS-induced afferent vagus nerve activation: possible role of nodose ganglion. Auton Neurosci 120:104-107.
Iijima N, Kataoka Y, Kakihara K, Bamba H, Tamada Y, Hayashi S, Matsuda T, Tanaka M, Honjyo H, Hosoya M, Hinuma S, Ibata Y (1999) Cytochemical study of prolactin-releasing peptide (PrRP) in the rat brain. Neuroreport 10:1713-1716.
Imeryuz N, Yegen BC, Bozkurt A, Coskun T, Villanueva-Penacarrillo ML, Ulusoy NB (1997) Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. American Journal of Physiology 273:G920-G927.
Iwai T, Ito S, Tanimitsu K, Udagawa S, Oka J (2006) Glucagon-like peptide-1 inhibits LPS-induced IL-1beta production in cultured rat astrocytes. Neuroscience research 55:352-360.
Jelsing J, Galzin A-M, Guillot E, Pruniaux M-P, Larsen PJ, Vrang N (2009) Localization and phenotypic characterization of brainstem neurons activated by rimonabant and WIN55,212-2. Brain Res Bull 78:202-210.
Johansson A, Fredriksson R, Winnergren S, Hulting AL, Schioth HB, Lindblom J (2008) The relative impact of chronic food restriction and acute food deprivation on plasma hormone levels and hypothalamic neuropeptide expression. Peptides 29:1588-1595.
Johnson AK, Epstein AN (1975) The cerebral ventricles as the avenue for the dipsogenic action of intracranial angiotensin. Brain Res 86:399-418.

Kachidian P, Pickel VM (1993) Localization of tyrosine hydroxylase in neuronal targets and efferents of the area postrema in the nucleus tractus solitarii of the rat. J Comp Neurol 329:337-353.
Kalia M, Sullivan JM (1982) Brainstem projections of sensory and motor components of the vagus nerve in the rat. J Comp Neurol 211:248-265.
Kalogeris TJ, Reidelberger RD, Mendel VE (1983) Effect of nutrient density and composition of liquid meals on gastric emptying in feeding rats. Am J Physiol 244:R865-871.
Kaneta T, Kusnecov AW (2005) The role of central corticotropin-releasing hormone in the anorexic and endocrine effects of the bacterial T cell superantigen, Staphylococcal enterotoxin A. Brain Behav Immun 19:138-146.
Kanoski SE, Fortin SM, Arnold M, Grill HJ, Hayes MR (2011) Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. Endocrinology 152:3103-3112.
Kanoski SE, Rupprecht LE, Fortin SM, De Jonghe BC, Hayes MR (2012) The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. Neuropharmacology 62:1916-1927.
Kasparov S, Teschemacher AG (2008) Altered central catecholaminergic transmission and cardiovascular disease. Exp Physiol 93:725-740.
Kawano H, Masuko S (1996) Neurons in the caudal ventrolateral medulla projecting to the paraventricular hypothalamic nucleus receive synaptic inputs from the nucleus of the solitary tract: a light and electron microscopic double-labeling study in the rat. Neurosci Lett 218:33-36.
Kinzig KP, D'Alessio DA, Seeley RJ (2002) The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci 22:10470-10476.
Kinzig KP, Hargrave SL, Honors MA (2008) Binge-type eating attenuates corticosterone and hypophagic responses to restraint stress. Physiol Behav 95:108-113.
Kinzig KP, D'Alessio DA, Herman JP, Sakai RR, Vahl TP, Figueiredo HF, Murphy EK, Seeley RJ (2003) CNS glucagon-like peptide-1 receptors mediate endocrine and anxiety responses to interoceptive and psychogenic stressors. J Neurosci 23:6163-6170.
Kissileff HR (1970) Free feeding in normal and "recovered lateral" rats monitored by a pellet-detecting eatometer. Physiol Behav 5:163-173.
Kmiec Z, Pokrywka L, Kotlarz G, Mysliwski A (2006) The effects of fasting and refeeding on adrenal cortex morphometry and serum concentrations of ACTH and corticosterone in young and old male rats. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 57 Suppl 6:77-84.
Kmiec Z, Pokrywka L, Kotlarz G, Kubasik J, Szutowicz A, Mysliwski A (2005) Effects of fasting and refeeding on serum leptin, adiponectin and free fatty acid concentrations in young and old male rats. Gerontology 51:357-362.
Kraly FS, Carty WJ, Smith GP (1978) Effect of pregastric food stimuli on meal size and internal intermeal in the rat. Physiol Behav 20:779-784.
Kreisler A, Rinaman L (2012) Co-activation of glucagon-like peptide-1 (GLP-1) and caudal A2 noradrenergic (NA) neurons in rats after rapid intake of a satiating meal. Society for Neuroscience Abstracts 93.15.
Kreisler AD, Davis EA, Rinaman L (2013) Differential activation of chemically identified neurons in the caudal nucleus of the solitary tract in non-entrained rats after intake of satiating vs. non-satiating meals. Physiol Behav 136:47-54.

Kyrou I, Tsigos C (2009) Stress hormones: physiological stress and regulation of metabolism. Current Opinion in Pharmacology 9:787-793.
Lachey JL, D'Alessio DA, Rinaman L, Elmquist JK, Drucker DJ, Seeley RJ (2005) The role of central glucagon-like peptide-1 in mediating the effects of visceral illness: differential effects in rats and mice. Endocrinology 146:458-462.
Langhans W, Balkowski G, Savoldelli D (1991) Differential feeding responses to bacterial lipopolysaccharide and muramyl dipeptide. Am J Physiol 261:R659-664.
Langhans W, Savoldelli D, Weingarten S (1993) Comparison of the feeding responses to bacterial lipopolysaccharide and interleukin-1 beta. Physiol Behav 53:643-649.
Langhans W, Harlacher R, Balkowski G, Scharrer E (1990) Comparison of the effects of bacterial lipopolysaccharide and muramyl dipeptide on food intake. Physiol Behav 47:805-813.
Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C (1997) Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. Neuroscience 77:257-270.
Lawrence CB, Ellacott KL, Luckman SM (2002) PRL-releasing peptide reduces food intake and may mediate satiety signaling. Endocrinology 143:360-367.
Lawrence CB, Celsi F, Brennand J, Luckman SM (2000) Alternative role for prolactin-releasing peptide in the regulation of food intake. Nat Neurosci 3:645-646.
Lawrence CB, Liu Y-L, Stock MJ, Luckman SM (2004) Anorectic actions of prolactin-releasing peptide are mediated by corticotropin-releasing hormone receptors. Am J Physiol Regul Integr Comp Physiol 286:R101-R107.
Li H-Y, Sawchenko PE (1998) Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: A comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels. J Comp Neurol 393:244-266.
Li H-Y, Ericsson A, Sawchenko PE (1996) Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. Proceedings of the National Academy of Sciences, USA 93:2359-2364.
Liposits Z, Phelix C, Paull WK (1986) Adrenergic innervation of corticotropin releasing factor (CRF) - synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. Histochemistry 84:201-205.
Llewellyn-Smith IJ, Reimann F, Gribble FM, Trapp S (2011) Preproglucagon neurons project widely to autonomic control areas in the mouse brain. Neuroscience 180:111-121.
Llewellyn-Smith IJ, Gnanamanickam GJ, Reimann F, Gribble FM, Trapp S (2013) Preproglucagon (PPG) neurons innervate neurochemically identified autonomic neurons in the mouse brainstem. Neuroscience 229:130-143.
Loewy AD (1990) Central autonomic pathways. In: Central Regulation of Autonomic Functions, 1 Edition (Loewy AD, Spyer KM, eds), pp 88-103. New York: Oxford University Press.
Luckman SM (1992) Fos-like immunoreactivity in the brainstem of the rat following peripheral administration of cholecystokinin. J Neuroendocrinol 4:149-152.
Luckman SM, Lawrence CB (2003) Anorectic brainstem peptides: more pieces to the puzzle. Trends in Endocrinology and Metabolism 14:60-65.
Lundy-Jr. RF, Norgren R (2004) Gustatory System. In: The Rat Nervous System, 3rd Edition (Paxinos G, ed), pp 890-921. San Diego: Elsevier Academic Press.

MacLusky NJ, Cook S, Scrocchi L, Shin J, Kim J, Vaccarino F, Asa SL, Drucker DJ (2000) Neuroendocrine function and response to stress in mice with complete disruption of glucagon-like peptide-1 receptor signaling. Endocrinology 141:752-762.

Maniscalco J, Gordon, P. J., Rinaman, L. (2014) Attenuated activation of glucagon-like peptide-1 (GLP-1)- and prolactin-releasing peptide (PrRP)- positive hindbrain neurons may contribute to fasting-mediated reductions in anxiety-like behavior and paraventricular hypothalamic (PVN) responses to cognitive stress. Society for Neuroscience Abstracts 452.12.

Maniscalco JW, Rinaman L (2013) Overnight food deprivation markedly attenuates hindbrain noradrenergic, glucagon-like peptide-1, and hypothalamic neural responses to exogenous cholecystokinin in male rats. Physiol Behav 121:35-42.

Maniscalco JW, Gordon PJ, Rinaman L (2012a) Activation of hindbrain glucagon-like peptide-1 (GLP-1) neurons in rats after restraint stress correlates with activation in the GLP-1 terminal-rich region of the parastrial nucleus (PS) of the hypotha- lamic preoptic area. . Society for Neuroscience Abstracts 684.13.

Maniscalco JW, Kreisler AD, Rinaman L (2012b) Satiation and stress-induced hypophagia: examining the role of hindbrain neurons expressing prolactin-releasing Peptide or glucagon-like Peptide 1. Front Neurosci 6:199.

Maniscalco JZ, H; Gordon, PJ; Rinaman, L. (in press) Negative energy balance blocks neural and behavioral responses to acute stress by "silencing" central glucagon-like peptide-1 (GLP-1) signaling in rats. J Neurosci.

Martinez-Peña-y-Valenzuela I, Rogers RC, Hermann GE, Travagli RA (2004) Norepinephrine effects on identified neurons of the rat dorsal motor nucleus of the vagus. American Journal of Physiology Gastrointestinal Liver Physiology 286:G333-G339.

Maruyama M, Matsumoto H, Fujiwara K, Noguchi J, Kitada C, Fujino M, Inoue K (2001) Prolactin-releasing peptide as a novel stress mediator in the central nervous system. Endocrinology 142:2032-2038.

Maruyama M, Matsumoto H, Fujiwara K, Kitada C, Hinuma S, Onda H, Fujino M, Inoue K (1999) Immunocytochemical localization of prolactin-releasing peptide in the rat brain. Endocrinology 140:2326-2333.

Matsumoto H, Maruyama M, Noguchi J, Horikoshi Y, Fujiwara K, Kitada C, Hinuma S, Onda H, Nishimura O, Inoue K, Fujino M (2000) Stimulation of corticotropin-releasing hormone-mediated adrenocorticotropin secretion by central administration of prolactin-releasing peptide in rats. Neurosci Lett 285:234-238.

McCann MJ, Verbalis JG, Stricker EM (1989) LiCl and CCK inhibit gastric emptying and feeding and stimulate OT secretion in rats. Am J Physiol 256:R463-468.

McMahon LR, Wellman PJ (1997) Decreased intake of a liquid diet in nonfood-deprived rats following intra-PVN injections of GLP-1 (7-36) amide. Pharmacol Biochem Behav 58:673-677.

McMahon LR, Wellman PJ (1998) PVN infusion of GLP-1-(7-36) amide suppresses feeding but does not induce aversion or alter locomotion in rats. Am J Physiol 274:R23-29.

Menétrey D, dePommery J (1991) Origins of spinal ascending pathways that reach central areas involved in visceroception and visceronociception in the rat. European Journal of Neuroscience 3:249-259.

Menétrey D, Basbaum, A. I. (1987) Spinal and trigeminal projections to the nucleus of the solitary tract: A possible substrate for somatovisceral and viscerovisceral reflex activaiton. J Comp Neurol 255:439-450.

Menétrey D, de Pommery J. (1990) Origins of spinal ascending pathways that reach central areas involved in visceroception and visceronociception in the rat. European Journal of Neuroscience 3:249-259.

Mera T, Fujihara H, Kawasaki M, Hashimoto H, Saito T, Shibata M, Saito J, Oka T, Tsuji S, Onaka T, Ueta Y (2006) Prolactin-releasing peptide is a potent mediator of stress responses in the brain through the hypothalamic paraventricular nucleus. Neuroscience 141:1069-1086.

Merchenthaler I, Lane M, Shughrue P (1999) Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J Comp Neurol 403:261-280.

Mizuno TM, Makimura H, Silverstein J, Roberts JL, Lopingco T, Mobbs CV (1999) Fasting regulates hypothalamic neuropeptide Y, agouti-related peptide, and proopiomelanocortin in diabetic mice independent of changes in leptin or insulin. Endocrinology 140:4551-4557.

Mochiduki A, Takeda T, Kaga S, Inoue K (2010) Stress response of prolactin-releasing peptide knockout mice as to glucocorticoid secretion. J Neuroendocrinol 22:576-584.

Morales T, Sawchenko PE (2003) Brainstem prolactin-releasing peptide neurons are sensitive to stress and lactation. Neuroscience 121:771-778.

Morales T, Hinuma S, Sawchenko PE (2000) Prolactin-releasing peptide is expressed in afferents to the endocrine hypothalamus, but not in neurosecretory neurones. J Neuroendocrinol 12:131-140.

Morley JE, Bartness TJ, Gosnell BA, Levine AS (1985) Peptidergic regulation of feeding. International Review of Neurobiology 27:207-298.

Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW (2006) Central nervous system control of food intake and body weight. Nature 443:289-295.

Myers EA, Rinaman L (2005) Trimethylthiazoline (TMT) supports conditioned flavor avoidance and activates viscerosensory, hypothalamic, and limbic circuits in rats. Am J Physiol Regul Integr Comp Physiol 288 R1716-R1726.

Myers EA, Banihashemi L, Rinaman L (2005) The anxiogenic drug yohimbine activates central viscerosensory circuits in rats. J Comp Neurol 492:426-441.

Nakade Y, Tsukamoto K, Pappas TN, Takahashi T (2006a) Central glucagon-like peptide 1 delays solid gastric emptying via central CRF and peripheral sympathetic pathways in rats. Brain Res 1111:117-121.

Nakade Y, Tsuchida D, Fukuda H, Iwa M, Pappas TN, Takahashi T (2006b) Restraint stress augments postprandial gastric contractions but impairs antropyloric coordination in conscious rats. American Journal of Physiology Regulatory Integrative and Comparative Physiology 290:R616-R624.

Norgren R (1978) Projections from the nucleus of the solitary tract in the rat. Neuroscience 3:207-218.

Ohiwa N, Chang H, Saito T, Onaka T, Fujikawa T, Soya H (2007) Possible inhibitory role of prolactin-releasing peptide for ACTH release associated with running stress. Am J Physiol Regul Integr Comp Physiol 292:R497-504.

Onaka T (2004) Neural pathways controlling central and peripheral oxytocin release during stress. J Neuroendocrinol 16:308-312.
Onaka T, Takayanagi Y, Leng G (2010) Metabolic and stress-related roles of prolactin-releasing peptide. Trends in endocrinology and metabolism: TEM 21:287-293.
Onaka T, Luckman SM, Antonijevic I, Palmer JR, Leng G (1995) Involvement of the noradrenergic afferents from the nucleus tractus solitarii to the supraoptic nucleus in oxytocin release after peripheral cholecystokinin octapeptide in the rat. Neuroscience 66:403-412.
Pearson RJ, Gatti PJ, Sahibzada N, Massari VJ, Gillis RA (2007) Ultrastructural evidence for selective noradrenergic innervation of CNS vagal projections to the fundus of the rat. Auton Neurosci 136:31-42.
Petrov T, Krukoff TL, Jhamandas JH (1993) Branching projections of catecholaminergic brainstem neurons to the paraventricular hypothalamic nucleus and the central nucleus of the amygdala in the rat. Brain Res 609:81-92.
Plata-Salaman CR, Borkoski JP (1993) Centrally administered bacterial lipopolysaccharide depresses feeding in rats. Pharmacol Biochem Behav 46:787-791.
Plotsky PM, Cunningham-Jr. ET, Widmaier EP (1989) Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. Endocr Rev 10:437-458.
Price JL (2005) Free will versus survival: brain systems that underlie intrinsic constraints on behavior. J Comp Neurol 493:132-139.
Renner E, Puskas N, Dobolyi A, Palkovits M (2012) Glucagon-like peptide-1 of brainstem origin activates dorsomedial hypothalamic neurons in satiated rats. Peptides 35:14-22.
Renner E, Szabo-Meltzer KI, Puskas N, Toth ZE, Dobolyi A, Palkovits M (2010) Activation of neurons in the hypothalamic dorsomedial nucleus via hypothalamic projections of the nucleus of the solitary tract following refeeding of fasted rats. Eur J Neurosci 31:302-314.
Reyes BAS, Bockstaele EJV (2006) Divergent projections of catecholaminergic neurons in the nucleus of the solitary tract to limbic forebrain and medullary autonomic brain regions. Brain Res 1117:69-79.
Riche D, Pommery JD, Menetrey D (1990) Neuropeptides and catecholamines in efferent projections of the nuclei of the solitary tract in the rat. Journal of Comparative Neurology 293:399-424.
Rinaman L (1999a) Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus. Am J Physiol 277:R582-590.
Rinaman L (1999b) A functional role for central glucagon-like peptide-1 receptors in lithium chloride-induced anorexia. Am J Physiol 277:R1537-1540.
Rinaman L (2000) The Autonomic Nervous System. In: Neuroscience Secrets (Wong-Riley MTT, ed), pp 225-237. Philadelphia: Hanley & Belfus, Inc.
Rinaman L (2003) Hindbrain noradrenergic lesions attenuate anorexia and alter central cFos expression in rats after gastric viscerosensory stimulation. J Neurosci 23:10084-10092.
Rinaman L (2004) Hindbrain contributions to anorexia. Am J Physiol Regul Integr Comp Physiol 287:R1035-R1036.
Rinaman L (2007) Visceral sensory inputs to the endocrine hypothalamus. Front Neuroendocrinol 28:50-60.
Rinaman L (2010) Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. Brain Res 1350:18-34.

Rinaman L (2011) Hindbrain noradrenergic A2 neurons: diverse roles in autonomic, endocrine, cognitive, and behavioral functions. Am J Physiol Regul Integr Comp Physiol 300:R222-235.

Rinaman L, Levitt P (1993) Establishment of vagal sensorimotor circuits during fetal development in rats. J Neurobiol 24:641-659.

Rinaman L, Dzmura V (2007) Experimental dissociation of neural circuits underlying conditioned avoidance and hypophagic responses to lithium chloride. Am J Physiol Regul Integr Comp Physiol 293:R1495-1503.

Rinaman L, Card JP, Schwaber JS, Miselis RR (1989) Ultrastructural demonstration of a gastric monosynaptic vagal circuit in the nucleus of the solitary tract in rat. J Neurosci 9:1985-1996.

Rinaman L, Verbalis JG, Stricker EM, Hoffman GE (1993) Distribution and neurochemical phenotypes of caudal medullary neurons activated to express cFos following peripheral administration of cholecystokinin. J Comp Neurol 338:475-490.

Rinaman L, Hoffman GE, Stricker EM, Verbalis JG (1994) Exogenous cholecystokinin activates cFos expression in medullary but not hypothalamic neurons in neonatal rats. Brain Res Dev Brain Res 77:140-145.

Rinaman L, Stricker EM, Hoffman GE, Verbalis JG (1997) Central c-fos expression in neonatal and adult rats after subcutaneous injection of hypertonic saline. Neuroscience 79:1165-1175.

Rinaman L, Baker EA, Hoffman GE, Stricker EM, Verbalis JG (1998) Medullary c-Fos activation in rats after ingestion of a satiating meal. Am J Physiol 275:R262-268.

Rinaman L, Hoffman GE, Dohanics J, Le WW, Stricker EM, Verbalis JG (1995) Cholecystokinin activates catecholaminergic neurons in the caudal medulla that innervate the paraventricular nucleus of the hypothalamus in rats. J Comp Neurol 360:246-256.

Ritter RC, Covasa M, Matson CA (1999) Cholecystokinin: proofs and prospects for involvement in control of food intake and body weight. Neuropeptides 33:387-399.

Ritter S, Watts AG, Dinh TT, Sanchez-Watts G, Pedrow C (2003) Immunotoxin lesion of hypothalamically projecting norepinephrine and epinephrine neurons differentially affects circadian and stressor-stimulated corticosterone secretion. Endocrinology 144:1357-1367.

Rogers RC, Travagli RA, Hermann GE (2003) Noradrenergic neurons in the rat solitary nucleus participate in the esophageal-gastric relaxation reflex. Am J Physiol Regul Integr Comp Physiol 285:R479-489.

Roland BL, Sutton SW, Wilson SJ, Luo L, Pyati J, Huvar R, Erlander MG, Lovenberg TW (1999) Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. Endocrinology 140:5736-5745.

Rowland NE, Crews EC, Gentry RM (1997) Comparison of Fos induced in rat brain by GLP-1 and amylin. Regul Pept 71:171-174.

Rushing PA, Houpt TA, Henderson RP, Gibbs J (1997) High lick rate is maintained throughout spontaneous liquid meals in freely feeding rats. Physiol Behav 62:1185-1188.

Rybkin, II, Zhou Y, Volaufova J, Smagin GN, Ryan DH, Harris RB (1997) Effect of restraint stress on food intake and body weight is determined by time of day. Am J Physiol 273:R1612-1622.

Samson WK, Keown C, Samson CK, Samson HW, Lane B, Baker JR, Taylor MM (2003) Prolactin-releasing peptide and its homolog RFRP-1 act in hypothalamus but not in anterior pituitary gland to stimulate stress hormone secretion. Endocrine 20:59-66.

Sapolsky R, Rivier C, Yamamoto G, Plotsky P, Vale W (1987) Interleukin-1 stimulates the secretion of hypothalamic corticotropin-releasing factor. Science 238:522-524.

Sarkar S, Fekete C, Legradi G, Lechan RM (2003) Glucagon like peptide-1 (7-36) amide (GLP-1) nerve terminals densely innervate corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus. Brain Res 985:163-168.

Sawchenko PE (1983) Central connections of the sensory and motor nuclei of the vagus nerve. J Auton Nerv Syst 9:13-26.

Sawchenko PE (1988) Effects of catecholamine-depleting medullary knife cuts on corticotropin-releasing factor and vasopressin immunoreactivity in the hypothalamus of normal and steroid-manipulated rats. Neuroendocrinology 48:459-470.

Sawchenko PE, Swanson LW (1981) Central noradrenergic pathways for the integration of hypothalamic neuroendocrine and autonomic responses. Science 214:685-687.

Sawchenko PE, Swanson LW (1982) Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. J Comp Neurol 205:260-272.

Sawchenko PE, Pfeiffer SW (1995) Ultrastructural localization of inhibin b- and somatostatin-28-immunoreactivities in the paraventricular and supraoptic nuclei. Brain Res 694:233-245.

Sawchenko PE, Arias C, Bittencourt JC (1990) Inhibin beta, somatostatin, and enkephalin immunoreactivities coexist in caudal medullary neurons that project to the paraventricular nucleus of the hypothalamus. J Comp Neurol 291:269-280.

Sawchenko PE, Li H-Y, Ericsson A (2000) Circuits and mechanisms governing hypothalamic responses to stress: a tale of two paradigms. In: Progress in Brain Research (Mayer EA, Saper CB, eds), pp 61-78: Elsevier Science BV.

Sawchenko PE, Swanson LW, Grzanna R, Howe PRC, Bloom SR, Polak JM (1985) Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nculeus of the hypothalamus. J Comp Neurol 241:138-153.

Schafer MK, Day R, Cullinan WE, Chretien M, Seidah NG, Watson SJ (1993) Gene expression of prohormone and proprotein convertases in the rat CNS: a comparative in situ hybridization analysis. J Neurosci 13:1258-1279.

Schick RR, Zimmermann JP, vorm Walde T, Schusdziarra V (2003a) Peptides that regulate food intake: glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats. Am J Physiol Regul Integr Comp Physiol 284:R1427-1435.

Schick RR, Zimmermann JP, Walde Tv, Schusdziarra V (2003b) Glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats. Am J Physiol Regulatory Integrative Comp Physiol 284:R1427-R1435.

Schiltz JC, Sawchenko PE (2007) Specificity and generality of the involvement of catecholaminergic afferents in hypothalamic responses to immune insults. J Comp Neurol 502:455-467.

Scrocchi LA, Hill ME, Saleh J, Perkins B, Drucker DJ (2000) Elimination of glucagon-like peptide 1R signaling does not modify weight gain and islet adaptation in mice with combined disruption of leptin and GLP-1 action. Diabetes 49:1552-1560.

Scrocchi LA, Brown TJ, MaClusky N, Brubaker PL, Auerbach AB, Joyner AL, Drucker DJ (1996) Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. Nat Med 2:1254-1258.

Seal LJ, Small CJ, Dhillo WS, Kennedy AR, Ghatei MA, Bloom SR (2002) Prolactin-releasing peptide releases corticotropin-releasing hormone and increases plasma adrenocorticotropin via the paraventricular nucleus of the hypothalamus. Neuroendocrinology 76:70-78.

Seeley RJ, Grill HJ, Kaplan JM (1994) Neurological Dissociation of Gastrointestinal and Metabolic Contributions to Meal Size Control. Behav Neurosci 108:347-352.

Seeley RJ, Blake K, Rushing PA, Benoit S, Eng J, Woods SC, D'Alessio D (2000) The role of CNS glucagon-like peptide-1 (7-36) amide receptors in mediating the visceral illness effects of lithium chloride. J Neurosci 20:1616-1621.

Serrats J, Sawchenko PE (2006) CNS activational responses to staphylococcal enterotoxin B: T-lymphocyte-dependent immune challenge effects on stress-related circuitry. J Comp Neurol 495:236-254.

Seto K, Sasaki T, Katsunuma K, Kobayashi N, Tanaka K, Tack J (2008) Acotiamide hydrochloride (Z-338), a novel prokinetic agent, restores delayed gastric emptying and feeding inhibition induced by restraint stress in rats. Neurogastroenterol Motil 20:1051-1059.

Shapiro RE, Miselis RR (1985) The central organization of the vagus nerve innervating the stomach of the rat. J Comp Neurol 238:473-488.

Shiraishi I, Honma K, Honma S, Hiroshige T (1984) Ethosecretogram: relation of behavior to plasma corticosterone in freely moving rats. Am J Physiol 247:R40-45.

Shirohzu H, Okabe T, Gondo S, Tanaka T, Ohe K, Morinaga H, Kawate H, Nomura M, Takayanagi R, Nawata H, Yanase T (2008) Methylation of a conserved intronic CpG island of mouse SF-1 is associated with cell-specific expression of SF-1 in a culture system but not with tissue-specific expression. Biochem Biophys Res Commun 369:862-867.

Shughrue PJ, Lane, M.V., Merchenthaler, I. (1996) Glucagon-Like Peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. Endocrinology 137:5159-5162.

Smith G (1998) Satiation: From gut to brain. New York: Oxford University Press.

Smith GP (1996) The direct and indirect controls of meal size. Neurosci Biobehav Rev 20:41-46.

Smith GP (2000) The controls of eating: a shift from nutritional homeostasis to behavioral neuroscience. Nutrition 16:814-820.

Smith GP (2004) Feeding: control of eating. In: Elsevier's Encyclopedia of Neuroscience (3rd Edition), Third Edition Edition (Adelman G, Smith BH, eds). New York: Elsevier.

Spyer KM (1990) The central nervous organization of reflex circulatory control. In: Central Regulation of Autonomic Functions (Loewy AD, Spyer KM, eds), pp 168-188. New York: Oxford University Press.

Stornetta RL, Sevigny CP, Guyenet PG (2002) Vesicular glutamate transporter DNPI/VGLUT2 mRNA is present in C1 and several other groups of brainstem catecholaminergic neurons. J Comp Neurol 444:191-206.

Strubbe JH, Keyser J, Dijkstra T, Prins AJ (1986) Interaction between circadian and caloric control of feeding behavior in the rat. Physiol Behav 36:489-493.

Suzuki H, Hibi T (2010) Acotiamide (Z-338) as a possible candidate for the treatment of functional dyspepsia. Neurogastroenterol Motil 22:595-599.

Swanson L (2004) Structure of the Rat Brain. Amsterdam: Elsevier.
Taché Y, Martinez V, Million M, Wang L (2001) Stress and the Gastrointestinal Tract. III. Stress-related alterations of gut motor function: role of brain corticotropin-releasing factor receptors. American Journal of Physiology Gastrointestinal and Liver Physiology 280:G173-G177.
Takayanagi Y, Onaka T (2010) Roles of prolactin-releasing peptide and RFamide related peptides in the control of stress and food intake. Febs J 277:4998-5005.
Takayanagi Y, Matsumoto H, Nakata M, Mera T, Fukusumi S, Hinuma S, Ueta Y, Yada T, Leng G, Onaka T (2008) Endogenous prolactin-releasing peptide regulates food intake in rodents. J Clin Invest 118:4014-4024.
Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, Sheikh SP (1996) Central administration of GLP-1-(7-36) amide inhibits food and water intake in rats. Am J Physiol 271:R848-856.
Thiele TE, Seeley RJ, D'Alessio D, Eng J, Bernstein IL, Woods SC, van Dijk G (1998) Central infusion of glucagon-like peptide-1-(7-36) amide (GLP-1) receptor antagonist attenuates lithium chloride-induced c-Fos induction in rat brainstem. Brain Res 801:164-170.
Toth ZE, Zelena D, Mergl Z, Kirilly E, Varnai P, Mezey E, Makara GB, Palkovits M (2008) Chronic repeated restraint stress increases prolactin-releasing peptide/tyrosine-hydroxylase ratio with gender-related differences in the rat brain. J Neurochem 104:653-666.
Traub RJ, Sengupta JN, Gebhart GF (1996) Differential c-fos expression in the nucleus of the solitary tract and spinal cord following noxious gastric distention in the rat. Neuroscience 74:873-884.
Travagli RA, Hermann GE, Browning KN, Rogers RC (2006) Brainstem circuits regulating gastric function. Annu Rev Physiol 68:279-305.
Travers JB, Rinaman L (2002) Identification of lingual motor control circuits using two strains of pseudorabies virus. Neuroscience 115:1139-1151.
Travers JB, Dinardo LA, Karimnamazi H (1997) Motor and premotor mechanisms of licking. Neurosci Biobehav Rev 21:631-647.
Travers JB, Herman K, Travers SP (2010) Suppression of third ventricular NPY-elicited feeding following medullary reticular formation infusions of muscimol. Behav Neurosci 124:225-233.
Tucker DC, Saper CB, Ruggiero DA, Reis DJ (1987) Organization of central adrenergic pathways: I. Relationships of ventrolateral medullary projections to the hypothalamus and spinal cord. J Comp Neurol 259:591-603.
Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR (1996) A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379:69-72.
Uchida K, Kobayashi D, Das G, Onaka T, Inoue K, Itoi K (2010) Participation of the prolactin-releasing peptide-containing neurones in caudal medulla in conveying haemorrhagic stress-induced signals to the paraventricular nucleus of the hypothalamus. J Neuroendocrinol 22:33-42.
Uchoa ET, Sabino HA, Ruginsk SG, Antunes-Rodrigues J, Elias LL (2009) Hypophagia induced by glucocorticoid deficiency is associated with an increased activation of satiety-related responses. J Appl Physiol 106:596-604.

Uehara A, Sekiya C, Takasugi Y, Namiki M, Arimura A (1989) Anorexia induced by interleukin 1: involvement of corticotropin-releasing factor. Am J Physiol 257:R613-617.
Verbalis JG, Stricker EM, Robinson AG, Hoffman GE (1991) Cholecystokinin activates C-fos expression in hypothalamic oxytocin and corticotropin-releasing hormone neurons. J Neuroendocrinol 3:205-213.
Verbalis JG, Baker EA, Rinaman L, Stricker EM, Hoffman GE (1995) c-Fos activation of catecholaminergic neurons in the dorsal vagal complex after gastric distension or CCK administration in rats. Society for Neuroscience Abstracts.
Vrang N, Larsen PJ (2010) Preproglucagon derived peptides GLP-1, GLP-2 and oxyntomodulin in the CNS: role of peripherally secreted and centrally produced peptides. Progress in Neurobiology 92:442-462.
Vrang N, Grove K (2011) The brainstem preproglucagon system in a non-human primate (Macaca mulatta). Brain Res 1397:28-37.
Vrang N, Phifer CB, Corkern MM, Berthoud HR (2003) Gastric distension induces c-Fos in medullary GLP-1/2-containing neurons. Am J Physiol Regul Integr Comp Physiol 285:R470-478.
Vrang N, Hansen M, Larsen PJ, Tang-Christensen M (2007) Characterization of brainstem preproglucagon projections to the paraventricular and dorsomedial hypothalamic nuclei. Brain Res 1149:118-126.
Wan S, Coleman FH, Travagli RA (2007) Glucagon-like peptide-1 excites pancreas-projecting preganglionic vagal motoneurons. American Journal of Physiology Gastrointestinal and Liver Physiology 292:G1474-G1482.
Watkins LR, Goehler LE, Relton JK, Tartaglia N, Silbert L, Martin D, Maier SF (1995) Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. Neurosci Lett 183:27-31.
Watson RE, Jr., Wiegand SJ, Clough RW, Hoffman GE (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7:155-159.
Watts AG (1996) The impact of physiological stimuli on the expression of corticotropin-releasing hormone (CRH) and other neuropeptide genes. Front Neuroendocrinol 17:281-326.
West DB, Fey D, Woods SC (1984) Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats. Am J Physiol 246:R776-787.
West DB, Greenwood MR, Marshall KA, Woods SC (1987) Lithium chloride, cholecystokinin and meal patterns: evidence that cholecystokinin suppresses meal size in rats without causing malaise. Appetite 8:221-227.
Williams DL, Baskin DG, Schwartz MW (2009) Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety. Endocrinology 150:1680-1687.
Williams DL, Hyvarinen N, Lilly N, Kay K, Dossat A, Parise E, Torregrossa AM (2011) Maintenance on a high-fat diet impairs the anorexic response to glucagon-like-peptide-1 receptor activation. Physiol Behav 103:557-564.
Woods SC (1991) The eating paradox: how we tolerate food. Psychol Rev 98:488-505.
Woods SC (2002) The house economist and the eating paradox. Appetite 38:161-165.
Woods SC, Seeley RJ (2000) Adiposity signals and the control of energy homeostasis. Nutrition 16:894-902.
Woods SC, Ramsay DS (2000) Pavlovian influences over food and drug intake. Behav Brain Res 110:175-182.

Yamada T, Mochiduki A, Sugimoto Y, Suzuki Y, Itoi K, Inoue K (2009) Prolactin-releasing peptide regulates the cardiovascular system via corticotrophin-releasing hormone. J Neuroendocrinol 21:586-593.

Yamamoto H, Kishi T, Lee CE, Choi BJ, Fang H, Hollenberg AN, Drucker DJ, Elmquist JK (2003) Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. Journal Neurosci 23:2939-2946.

Yamashita H, Kannan H, Ueta Y (1989) Involvement of caudal ventrolateral medulla neurons in mediating visceroreceptive information to the hypothalamic paraventricular nucleus. Prog Brain Res 81:293-302.

Yano T, Iijima N, Kataoka Y, Hinuma S, Tanaka M, Ibata Y (2001) Developmental expression of prolactin releasing peptide in the rat brain: localization of messenger ribonucleic acid and immunoreactive neurons. Brain Res Dev Brain Res 128:101-111.

Young RC, Gibbs J, Antin J, Holt J, Smith GP (1974) Absence of satiety during sham feeding in the rat. J Comp Physiol Psychol 87:795-800.

Zagon A, Rocha I, Ishizuka K, Spyer KM (1999) Vagal modulation of responses elicited by stimulation of the aortic depressor nerve in neurons of the rostral ventrolateral medulla oblongata in the rat. Neuroscience 92:889-899.

Zhang R, Jankord R, Flak JN, Solomon MB, D'Alessio DA, Herman JP (2010) Role of glucocorticoids in tuning hindbrain stress integration. J Neurosci 30:14907-14914.

Zhang X, Fogel R, Renehan WE (1995) Relationships between the morphology and function of gastric- and intestine-sensitive neurons in the nucleus of the solitary tract. J Comp Neurol 363:37-52.

Zhang X, Cui J, Tan Z, Jiang C, Fogel R (2003) The central nucleus of the amygdala modulates gut-related neurons in the dorsal vagal complex in rats. Journal of Physiology 553:1005-1018.

Zhao S, Kanoski SE, Yan J, Grill HJ, Hayes MR (2012) Hindbrain leptin and glucagon-like-peptide-1 receptor signaling interact to suppress food intake in an additive manner. Int J Obes (Lond).

Zheng H, Stornetta RL, Agassandian K, Rinaman L (2014) Glutamatergic phenotype of glucagon-like peptide 1 neurons in the caudal nucleus of the solitary tract in rats. Brain Structure & Function.

Zhu LL, Onaka T (2003) Facilitative role of prolactin-releasing peptide neurons in oxytocin cell activation after conditioned-fear stimuli. Neuroscience 118:1045-1053.

Zittel TT, Glatzle J, Kreis ME, Starlinger M, Eichner M, Raybould HE, Becker HD, Jehle EC (1999) C-fos protein expression in the nucleus of the solitary tract correlates with cholecystokinin dose injected and food intake in rats. Brain Res 846:1-11.