Metabolic Tuning of Neural, Neuroendocrine, and Behavioral Responses to Stress

by

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Bachelor of Science, Denison University, 2009

Submitted to the Graduate Faculty of

The Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

The Dietrich School of Arts and Sciences

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METABOLIC TUNING OF NEURAL, NEUROENDOCRINE, AND BEHAVIORAL RESPONSES TO STRESS

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In response to actual or anticipated stressors, the central nervous system elicits neural, neuroendocrine, and behavioral stress responses that facilitate survival and restoration of homeostatic balance. Caloric deficit attenuates many of these responses, including neural drive to the hypothalamic-pituitary-adrenal (HPA) axis, anxiety-like behavior, and stress-induced hypophagia. These alterations may represent an adaptive shift in stress responses during periods of negative energy balance, however, the neural mechanisms by which they occur remain unclear. Hindbrain glucagon-like peptide-1 (GLP-1) neurons and prolactin-releasing peptide-positive noradrenergic A2 ($PrRP^+$ A2) neurons are stress-sensitive and receive robust ingestive/metabolic information. Moreover, signaling from these populations contributes to each of the stress responses altered during fasting. Considering this, we hypothesized that the ability of caloric deficit to decrease anxiety-like behavior, stress hypophagia, and central drive to the HPA axis depends on reduced signaling from hindbrain GLP-1 and $PrRP^+$ A2 neurons.

In Chapter 2, we demonstrate that overnight fasting markedly reduces visceral stress-induced cFos activation of GLP-1 neurons, A2 neurons, and forebrain neurons implicated in HPA axis drive. Furthermore, the results of Chapter 3 reveal that overnight fasting nearly eliminates cognitive stress-induced activation of GLP-1 and $PrRP^+$ A2 neurons. Fasting also decreased anxiety-like behavior, in conjunction with attenuated cognitive stress-induced cFos expression within a limbic forebrain nucleus implicated in anxiogenesis and HPA axis activation.

Finally, using central administration of a GLP-1 receptor antagonist, we demonstrated that central GLP-1 signaling is critical for cognitive stress-induced hypophagia. Together, these findings document the metabolic tuning of stress-induced GLP-1 and $PrRP^{+}$ A2 neural activation and implicate fasting-mediated reductions in activation of these neurons in the attenuation of behavioral stress responses. Considering this, we investigated the mechanisms by which fasting alters activation of GLP-1 and $PrRP^{+}$ A2 neurons. Our results revealed that systemic ghrelin receptor antagonist administration partially rescued stress-induced activation of GLP-1 and $PrRP^{+}$ A2 neurons in fasted rats. In sum, the results of this dissertation work support the hypothesis that metabolic tuning of hindbrain GLP-1 and $PrRP^{+}$ A2 neurons is a mechanism through which caloric deficit decreases central drive to the HPA axis, promotes anxiolysis, and attenuates hypophagic responses to acute stress.

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PREFACE

Acknowledgements

Researching and writing a doctoral dissertation is in many ways a group effort, and I have many people to thank for their indispensable help and support during my graduate training. First of all, I would like to thank all of the members of the Center for Neuroscience at the University of Pittsburgh, and in particular those in the Department of Neuroscience; it has been an honor and a pleasure to conduct my graduate training in the warm, supportive, and intellectually stimulating environment fostered by the members of these communities.

To the fantastic staff members and administrators in the Department of Neuroscience, thank you so much for everything you did to keep me on track to graduate since my entry into the program. In particular, I'd like to thank Joan Blaney; Joan, you are an absolutely wonderful administrator and friend, and I am so glad to have had the opportunity to work with you. It was always a pleasure stopping by to see you, and I certainly would not have graduated without all of your help and hard work.

I would also like to thank the many friends I have made while in Pittsburgh; you have influenced me personally and professionally in so many ways, and I wish I could thank each of you individually for your contribution to the success of my doctoral work. Michelle and Nicole, it has been great to learn alongside you and to grow together as scientists over the past five years. You have each been an inspiration to me, and I'm so thankful that we became such good friends

during our graduate training. Laura and Rachel, it's been a pleasure getting to know each of you over the past few years, and I can't thank you enough for your friendship and support though this entire process.

I would like to thank all of the members of the Rinaman Lab, past and present, for creating a wonderful environment in which to work and train. Vicki, Huiyuan, and Li, thank you for all of your technical support and your friendship over the past five years. Layla and Mike, you have been terrific role models and friends, and I am so thankful for everything you've taught me since I joined the Rinaman Lab. Alison, it's been a great experience working alongside you, teaching you, and learning from you. Your friendship and scientific/moral support were critical for the successful completion of my thesis work, and I'm so appreciative of everything you've done for me.

To my internal committee members – Alan, Michael, and Judy – thank you for all of your support and input over the past few years, I truly appreciate your commitment to my graduate training and the time and effort you put into making my dissertation successful. Jim, thank you for your willingness to participate as my outside examiner, it was a pleasure having you on my committee and getting to discuss our shared scientific interests. Pat, my dissertation committee chair, since the day I walked into your office to interview for the CNUP graduate program, you have been a great source of support and friendship. Not only have you participated on most of my graduate school committees, but you've also been someone I could rely on for advice, insight, and friendly conversation. Thank you for everything you've done to make my graduate training successful.

Linda, thank you for being such a fantastic mentor and friend; I couldn't have asked for a better advisor. You have shaped my intellectual and personal development in more ways than

you know, and I am so grateful for your commitment to my training and success. It has been an honor and a pleasure to work in your lab, and I am so very thankful for everything you have done for me over the past five years.

The biggest of acknowledgements to my parents; Mom and Dad, I would not be who I am today – and I would not have completed my PhD – without your love and support. I can't thank you enough for everything you've done to allow me to succeed in life, and I will be forever grateful. I love you both. Finally, to my wonderful wife Stacey: thank you, thank you, thank you. I am so lucky to be with you, and I certainly could not have earned my PhD without all of your love, support, and hard work. Thank you so much for keeping our lives organized and on track, for making sure we had some fun, excitement, and relaxation (even at the toughest junctures), and for keeping me sane through the dissertation writing process. You have always pushed me to be a better person, both personally and professionally, and I can't tell you how much I appreciate that. I love you.

List of Abbreviations

ACTH – adrenocorticotropic hormone

Ad lib – *ad libitum*

AgRP – agouti-related protein

AngII – angiotensin II

ANOVA – analysis of variance

AP – area postrema

ARC – arcuate nucleus of the hypothalamus

BBB – blood brain barrier

(vl)BST – (ventrolateral) bed nucleus of the stria terminalis

BW – body weight

CART – cocaine- and amphetamine-regulated transcript

CCK – cholecystokinin-8

CeA – central nucleus of the amygdala

ChR2 – channelrhodopsin-2

CNS – central nervous system

CORT – corticosterone

CRH – corticotropin-releasing hormone

cVLM – caudal ventrolateral medulla

(Ni)DAB – diaminobenzidine (intensified with nickel sulfate)

DEP – overnight food-deprived

DMX/DMV – dorsal motor nucleus of the vagus nerve

DREADD – designer receptors exclusively activated by designer drugs

DVC – dorsal vagal complex

DβH – dopamine-β-hydroxylase

EP – elevated platform

EPMZ – elevated plus maze

Ex9/Ex-9 – exendin-(9-39)

GC(s) – glucocorticoid(s)

GI – gastrointestinal

GLP-1 – glucagon-like peptide-1

GLP-1R – glucagon-like peptide-1 receptor

GRA – ghrelin receptor antagonist

GRs – glucocorticoid receptors

HPA – hypothalamic-pituitary-adrenal

i.c.v. – intracerebroventricular

i.p. – intraperitoneal or intraperitoneally

ir – immunoreactivity or immunoreactive

JAK-STAT – janus kinase/signal transducer and activator of transcription

LiCl – lithium chloride

LPS – lipopolysaccharide

NA – noradrenergic

NAcc – nucleus accumbens

NE – norepinephrine

NH – nonhandled

NPY – neuropeptide Y

(c)NTS / (c)NST – (caudal) nucleus of the solitary tract

mNTS – nucleus of the solitary tract (medial subnucleus)

OLETF – Otsuka Long-Evans Tokushima Fatty

(mp)PVN – (medial parvocellular) paraventricular nucleus of the hypothalamus

POMC – pro-opiomelanocortin

PPG – preproglucagon

PrRP – prolactin-releasing peptide

RES – restraint

ROI – region of interest

Sal – saline

(p)STAT3 – (phosphorylated) signal transducer and activator of transcription 3

SF-1 – steroidogenic factor-1

SNS – sympathetic nervous system

TH – tyrosine hydroxylase

vGlut2 – vesicular glutamate transporter 2

cVMH – ventromedial hypothalamus (central subregion)

dmVMH – ventromedial hypothalamus (dorsomedial subregion)

vlVMH – ventromedial hypothalamus (ventrolateral subregion)

VTA –ventral tegmental area

1.0 INTRODUCTION

1.1 HOMEOSTASIS, STRESS, AND CENTRAL NERVOUS SYSTEM-MEDIATED STRESS RESPONSES

Despite marked variation in environmental conditions, the internal milieu of mammals is maintained in a state of dynamic equilibrium known as homeostasis. The maintenance of homeostasis is necessary for survival and well-being, as homeostatic regulation influences a number of critical physiological outcomes, such as body temperature, blood pressure, and blood glucose levels. Homeostasis is frequently challenged by internal and external forces, referred to as stressors, which can be as salient as an attack by a predator or as subtle as a drop in blood pressure when moving from a seated to standing position (Sapolsky, 2004). In response to stressors, the central nervous system (CNS) elicits a constellation of neural, neuroendocrine, and behavioral responses that facilitate immediate survival and eventual restoration of homeostatic balance. These stress responses provide an important means by which to adapt the physiological and neural state to the frequent homeostatic threats that pervade daily life. Hyperactivity of centrally-mediated stress responses (i.e., responses of increased duration and/or magnitude), however, has been linked to numerous neuropsychiatric, neurological, and physiological diseases and disorders, emphasizing the need to better understand how the brain coordinates responses to acute and chronic stress exposure (Chrousos, 2009).

Importantly, central stress responses are malleable, and can be modified and reorganized based on current physical state and/or prior experience (Bhatnagar and Dallman, 1998; Ma and Morilak, 2005; Zhang et al., 2010). Caloric deficit (i.e., periods of food restriction or deprivation) substantially attenuates many centrally-mediated responses to stress, including neural drive to the hypothalamic-pituitary-adrenal (HPA) axis (Hanson et al., 1994; Kiss et al., 1994), anxiety-like behavior (Genn et al., 2003; Inoue et al., 2004), and stress-induced suppression of food intake (a.k.a., stress-induced hypophagia) (Lennie et al., 1995; Youngblood et al., 1997). It has been posited that this reorganization decreases the ability of stressors to reduce exploration, foraging behavior, and food intake during periods of negative energy balance, thus increasing the likelihood of finding food and repleting energy stores (Dallman et al., 1999; Genn et al., 2003). The mechanisms by which caloric deficit alters central stress responses, however, remain unclear. The overarching aim of the research presented within this dissertation is to establish foundational evidence that a brief period of complete food deprivation alters central stress responses by attenuating activation of stress-sensitive neurons residing in the hindbrain, providing insights into the mechanisms by which metabolic state elicits widespread reconfiguration of centrally-mediated stress responses.

1.2 THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

The HPA axis is the major neuroendocrine component of the stress response. The apex of the HPA axis lies within corticotropin-releasing hormone (CRH) neurons of the medial parvocellular paraventricular nucleus of the hypothalamus (mpPVN). Activation of these neurons causes CRH release into the hypophyseal portal system, which circulates CRH into the anterior pituitary

gland. Here, CRH acts at corticotropes to cause systemic release of adrenocorticotropic hormone (ACTH), which results in corticosterone (CORT) synthesis in the adrenal cortex (Chrousos, 1998). CORT, a steroid hormone, then acts at glucocorticoid receptors (GRs) throughout the body. CORT has many adaptive effects that facilitate survival in the face of actual or anticipated environmental insults, of which energy mobilization is arguably the most important (Sapolsky et al., 2000). In conjunction with activation of the sympathetic nervous system (SNS), CORT facilitates muscular and hepatic glycogenolysis to mobilize energy substrates, while stimulating gluconeogenesis to maintain/elevate circulating glucose. Synthesis and mobilization of glucose is critical to fuel numerous energy-demanding stress responses, notably the skeletal muscle contractions integral for the "fight or flight" response. Concurrently, CORT functions to suppress energy expensive, long-term processes, such as immune system function and growth, shifting the allocation of resources to processes adaptive for immediate survival (Sapolsky, 2004). Activation of the HPA axis is regulated by a complex network of central circuits, all of which converge on hypophysiotropic neurons of the mpPVN (Ulrich-Lai and Herman, 2009), and major changes in the neuroendocrine response can be elicited by influencing individual or multiple components of these circuits.

1.3 BEHAVIORAL STRESS RESPONSES

In addition to the neural and physiological responses critical for survival following stress exposure, threatening and stressful stimuli elicit a number of adaptive behavioral responses. In rodents, behavioral responses to stress can be characterized generally by inhibition. Exploration and food intake represent a substantial contribution to the rodent behavioral repertoire, and each

is adaptive when considered in the context of maintaining internal energy and nutrient availability. Following stress exposure, however, these responses can each be considered maladaptive: exploration of novel environments may increase the likelihood of predation, while food intake initiates the energetically-expensive process of digestion, diverting energy resources away from functions with more immediate value. Thus, it is not surprising that a wide range of stressors cause increased behavioral anxiety and decreased food intake (i.e., hypophagia).

1.3.1 Anxiety-like behavior

In rodents, anxiety is characterized by behavioral avoidance, hyper-vigilance, and increased attention, which depend on the output of widely distributed neural circuits (Walker et al., 2003). An accumulated literature has implicated the bed nucleus of the stria terminalis (BST) and the central nucleus of the amygdala (CeA), collectively referred to as the "central extended amygdala," in the coordination of these responses. The CeA and BST receive neural input from the cortex, hippocampus, brainstem, and other amygdalar subnuclei (e.g., the basolateral amygdala) that process stimuli associated with stress and fear (Bienkowski and Rinaman, 2013). In turn, the BST and CeA convey this information to widespread target areas that mediate individual components of the anxiety state (Dong et al., 2001; Walker et al., 2003). This connectivity optimally positions the BST and CeA to integrate diverse information regarding stressful/anxiogenic stimuli and produce behavioral responses to both internal and external threats.

Behavioral and pharmacological studies substantiate the role of the central extended amygdala in anxiety-like behavior, as lesions or pharmacological inactivation of the CeA or BST produce anxiolytic effects, as measured by behavioral avoidance or freezing/startle responses

(Sullivan et al., 2004; Waddell et al., 2006; Sajdyk et al., 2008; Ventura-Silva et al., 2013). Work by Davis and colleagues suggests that the CeA is critical for eliciting short-lasting, stimulus-specific responses to conditioned threat cues (i.e., fear responses), while neurons of the BST are critical for eliciting sustained behavioral responses to innate, unconditioned threatening stimuli, such as those to open spaces, bright light, and predator odor (i.e., anxiety responses) (Lee and Davis, 1997; Walker and Davis, 1997; Fendt et al., 2003; Walker et al., 2003). Thus, by modulating neural activity in the BST, it is possible to modify the behavioral responses associated with a sustained state of anxiety.

1.3.2 Suppression of food intake

Suppression of food intake is a well-documented response to a wide array of acute stressors (Uehara et al., 1989; Rybkin et al., 1997; Liu et al., 2007; Calvez et al., 2011; Maniam and Morris, 2012); however, the neural mechanisms by which this phenomenon occurs are not entirely clear. While stress-induced suppression of food intake (i.e., stress hypophagia) can be broadly considered as a decrease in food intake over time, factors that change intake may do so by altering meal size, meal frequency, or both (Smith, 1998; Smith, 2000), and an understanding of the way in which stress decreases food intake can provide important information regarding the neural mechanisms likely to be involved. Diverse stressors, such as restraint, forced swimming, and (LiCl) chloride administration, elicit hypophagia via decreases in meal size (Dess and Vanderweele, 1994; Calvez et al., 2011). As satiation – the process that determines meal size – is a brainstem-mediated phenomenon (Grill and Norgren, 1978; Seeley et al., 1994; Grill and Kaplan, 2002; Grill, 2010), it is likely that stress-induced hypophagia results from influence on brainstem circuits responsible for satiation.

Following food intake, the hormonal and mechanical indices of nutrient and caloric presence in the gut are transmitted to the caudal ("visceral") nucleus of the solitary tract (cNTS) predominantly via direct inputs from vagal afferent sensory neurons (Rinaman, 2007). In the cNTS, glutamatergic vagal afferent signaling produces tightly synced, large-amplitude excitatory postsynaptic currents, providing high-fidelity transmission of sensory nerve activity (Appleyard et al., 2007). Activation of cNTS neurons then restricts meal size via regulation of pre-oral motor neurons within the brainstem parvocellular and intermediate reticular formation (Nasse et al., 2008). These reticular neurons maintain efferent synaptic connections with motor neurons innervating muscles of the tongue (Travers and Rinaman, 2002; Travers et al., 2005) and other orofacial targets to directly control the motor patterns leading to ingestion or rejection of oral contents (Chen et al., 2001), thus determining meal size. In essence, these brainstem neurons produce a complex reflex arc by which ingestion of a meal provides feedback to the motor neurons responsible for the process of food intake.

Although neural processing within the brainstem is sufficient for satiation (Grill and Smith, 1988), direct descending projections from the cortex, limbic forebrain, and hypothalamus to the cNTS provide a route through which emotional and cognitive events can modulate initiation or avoidance of feeding and other responses to diverse threats, including conditioned responses that are based on past experience (Li et al., 1996; Li and Sawchenko, 1998; Woods and Ramsay, 2000; Dayas and Day, 2001; Taché et al., 2001; Buller et al., 2003; Dayas et al., 2004; Blevins and Baskin, 2010; Grill and Hayes, 2012). Considered together, it appears likely that stressors elicit hypophagia via influence over brainstem satiation circuits.

1.4 VISCERAL AND COGNITIVE STRESSORS

HPA axis activation, anxiety-like behavior, and hypophagia are consistent responses to a wide array of stressors. The neural circuits by which these responses are elicited, however, vary considerably based on the stimulus attributes of the stressor (Herman and Cullinan, 1997), which has led to the categorization of stressors into visceral and cognitive categories.

1.4.1 Visceral stressors

Visceral (a.k.a. interoceptive or systemic) stressors represent a class of stimuli that have already disrupted homeostasis (e.g., infection, hemorrhage, hypoxia) and present an immediate threat to survival. Visceral stressors originate within the body and signals from stressful visceral stimuli are conveyed to the cNTS via direct or indirect sensory input. Predominant sources of cNTS sensory input arise from spinal afferents, area postrema (AP) sensory neurons, and the vagus and glossopharyngeal cranial nerves, which ramify throughout a majority of peripheral structures (Rinaman, 2007). These inputs are responsive to signals of visceral stress and their axons converge onto cNTS neurons that are positioned to 1) suppress food intake, via influence over brainstem reticular neurons (Grill et al., 2004), 2) to activate the HPA axis, via direct innervation of the mpPVN (Rinaman, 2010), and 3) to elicit behavioral anxiety, via dense projections to the BST (Banihashemi and Rinaman, 2006). Thus, adaptive neuroendocrine and behavioral anxiety responses to visceral stressors are contingent upon signaling between afferent sensory inputs, the cNTS, and the forebrain, while visceral stress-induced hypophagia can be mediated by cNTS projections to local brainstem circuits (Figure 1).

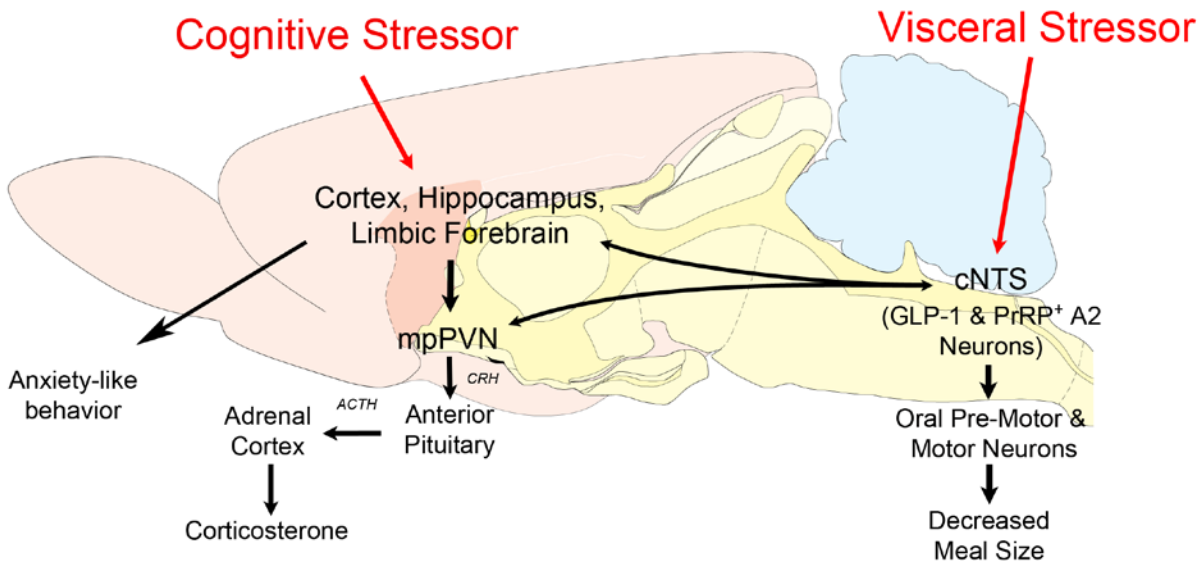

Figure 1. Schematic representation of selected stress-sensitive neural circuits and their putative relationships with behavioral and neuroendocrine stress responses.

cNTS, caudal nucleus of the solitary tract; GLP-1, glucagon-like peptide-1; $PrRP^{+}$ A2, prolactin-releasing peptide-positive noradrenergic A2; mpPVN, medial parvocellular paraventricular nucleus of the hypothalamus. Note: "limbic forebrain" includes the bed nucleus of the stria terminalis and the central nucleus of the amygdala.

1.4.2 Cognitive stressors

Cognitive (a.k.a. psychogenic or processive) stressors represent a class of stimuli that have not yet produced an actual physical challenge, but rather indicate imminent disruption of homeostasis based on instinctual or learned processes (e.g., the odor of a predator, isolation in a threatening environment, a tone that has been previously paired with an electrical shock). Complex and anticipatory in nature, responses to cognitive stressors require neural processing by regions responsible for integration of multiple sensory modalities (e.g., the cortex and thalamus), executive function (e.g. the medial prefrontal cortex), and memory (e.g. the hippocampus) before eliciting changes in neuroendocrine, autonomic, or behavioral output (Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009), largely via relays in the medial and baslolateral amygdalar subnuclei (Figure 1) (Dunn and Whitener, 1986; Cullinan et al., 1995; Dayas et al., 1999; LeDoux, 2000; Dayas et al., 2001b). Cognitive stressors also activate neurons within the cNTS, in part through descending projections from the PVN (Dayas and Day, 2001; Dayas et al., 2004), and we propose that these descending projections underlie the ability of cognitive stressors to suppress food intake. Interestingly, neuroendocrine responses to cognitive stressors require recruitment of mpPVN-projecting cNTS neurons (Kinzig et al., 2003), placing this nucleus in a position to influence both visceral and cognitive stress responses (Figure 1).

1.5 CALORIC DEFICIT ALTERS STRESS RESPONSES

In rodents, periods of food restriction or fasting (i.e., caloric deficit) substantially attenuate neurally-mediated stress responses. Chronic food restriction for a period of days to weeks, in which daily caloric intake is limited to reduce body weight to a defined degree, consistently decreases behavioral measures of anxiety in rodents (Heiderstadt et al., 2000; Genn et al., 2003; Levay et al., 2007). Interestingly, the anxiolytic effect of caloric deficit can be observed following a single day of restricted access to food (Inoue et al., 2004), suggesting that the neurophysiological changes mediating this shift in behavior occur rapidly. Similar effects of caloric restriction on stress-induced suppression of food intake have been observed. Chronic caloric restriction prevents restraint stress, a robustly hypophagic cognitive stressor in *ad libitum* (ad lib)-fed rats (Krahn et al., 1986), from decreasing food intake (Youngblood et al., 1997). Caloric deficit is also sufficient to decrease hypophagic responses to an acute inflammatory event (Lennie et al., 1995) or cholecystokinin treatment (McMinn et al., 2000), indicating that caloric deficit reduces the ability of both cognitive and visceral stressors to suppress food intake.

Consistent with these findings, chronic caloric restriction, prolonged periods of complete fasting (2-4 days), or a single overnight fast attenuate central drive to the HPA axis, as evidenced by decreased mpPVN CRH mRNA expression (Brady et al., 1990; Kiss et al., 1994), decreased baseline mpPVN cFos activation (Dallman et al., 1999), and decreased plasma ACTH concentrations at baseline and in response to restraint stress (Akana et al., 1994; Hanson et al., 1994; Chacon et al., 2005). Paradoxically, caloric deficit increases basal and restraint stress-induced plasma CORT levels in these same animals (Akana et al., 1994; Chacon et al., 2005), evidence that fasting “uncouples” neurally-mediated behavioral stress responses (i.e., anxiety and hypophagia) and ACTH release from adrenocortical CORT responses.

It is posited that the benefits of this uncoupling are three-fold. First, since central CRH signaling has potent anorexigenic effects (Rothwell, 1990) and contributes to hypophagic responses to stress exposure (Krahn et al., 1986), downregulating central CRH signaling may attenuate stress hypophagia, limiting the ability of stress to suppress food intake during periods of negative energy balance. Second, as CORT signaling stimulates glycogenolysis, energy substrate mobilization, and gluconeogenesis, increased circulating CORT concentrations during fasting may facilitate energy mobilization to support glucose-dependent processes, despite negative energy balance (Dallman et al., 1999). Third, attenuated anxiety may promote foraging in threatening environments, increasing the likelihood of encountering food. Together, these alterations represent an adaptive reorganization of stress responses during caloric deficit, catering the response to the animal's current metabolic state; however the neural mechanisms by which this shift occurs remain unclear.

We hypothesize that the ability of caloric deficit to decrease stress hypophagia, anxiety-like behavior, and central drive to the HPA axis depends on reduced signaling from prolactin-releasing peptide (PrRP)-positive noradrenergic (NA) A2 neurons ($PrRP^+$ A2 neurons) and glucagon-like peptide-1 (GLP-1)-positive neurons in the cNTS and medullary reticular formation. These neural populations are stress-sensitive, responsive to changes in the interoceptive environment, and have been implicated anatomically and functionally in HPA axis activation, anxiety-like behavior, and stress hypophagia.

1.6 STRESS RESPONSIVE HINDBRAIN NEURONS

1.6.1 PrRP$^+$ A2 neurons & hindbrain GLP-1 neurons: Anatomy & stress-sensitivity

The A2 cell group is composed of NA neurons that are located entirely within the NTS, and predominantly within the medial and commissural NTS subnuclei. The A2 cell group extends from the upper cervical spinal cord to just rostral to the AP at the level of the caudal fourth ventricle (Rinaman, 2011). These neurons can be identified by immunolabeling for tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, as well as dopamine-β-hydroxylase (DβH), the enzyme responsible for conversion of dopamine to norepinephrine (NE). A majority of A2 neurons co-localize PrRP (Roland et al., 1999; Maruyama et al., 2001), a neuropeptide originally named for its stimulatory effect on prolactin release from the anterior pituitary *in vitro* (Hinuma et al., 1998). More recent studies indicate that PrRP$^+$ A2 neurons do not have access to prolactin cells of the anterior pituitary (Morales et al., 2000) and PrRP signaling does not alter prolactin release *in vivo* (Taylor and Samson, 2001).

GLP-1 neurons reside within both the cNTS and subjacent reticular formation (Larsen et al., 1997b; Rinaman, 1999b, 2003a), extending from the upper cervical spinal cord to the caudal level of the AP (Vrang and Larsen, 2010). Despite the largely overlapping hindbrain distribution of A2 neurons 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 (Figure 20). Within the brain, PPG mRNA expression is limited to the olfactory bulb, the cNTS, and the caudal medullary reticular formation (Larsen et al., 1997b; Merchenthaler et al., 1999). 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 PPG-expressing hindbrain neurons.

The cNTS is considered the "visceral" NTS, distinct from the more rostral "gustatory" NST (Lundy-Jr. and Norgren, 2004), and is a key component of the dorsal vagal complex (DVC), which also includes the AP and dorsal motor nucleus of the vagus. The DVC is a critical central node for relaying interoceptive visceral, hormonal, and somatic feedback from body to brain, and regulating glucose homeostasis and other aspects of energy balance (Zagon et al., 1999; Berthoud et al., 2006; Rinaman, 2007; Grill and Hayes, 2009; 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, and AP neurons innervate the cNTS (Shapiro and Miselis, 1985a; Kachidian and Pickel, 1993; Cunningham-Jr. et al., 1994), allowing bloodborne factors to affect neurons in this region (Yamamoto et al., 2003). Sensitivity to circulating factors, in addition to robust spinal and cranial nerve afferent innervation (Sections 1.3.2 & 1.4.1), positions the cNTS to integrate multimodal input regarding metabolic state and to monitor changes in the internal environment.

Numerous studies show that cNTS $PrRP^{+}$ A2 and GLP-1 neurons are activated by a broad array of visceral stressors, including: hemorrhage (Morales and Sawchenko, 2003; Uchida et al., 2010), high doses of cholecystokinin-8 (CCK) (Rinaman, 1999b; Lawrence et al., 2002), lipopolysaccharide (LPS) (Mera et al., 2006), lithium chloride (Rinaman, 1999b), and interleukin-1 (Li et al., 1996; Sawchenko et al., 2000). Notably, $PrRP^{+}$ A2 neurons are also activated by cognitive stressors, including: restraint (Dayas et al., 2001b; Maruyama et al., 2001; Banihashemi et al., 2011), footshock (Li et al., 1996; Morales and Sawchenko, 2003), predator odor (Day et al., 2004), and conditioned fear cues (Zhu and Onaka, 2003). Activation of cNTS

neurons by cognitive stressors is likely a result of descending projections from limbic and hypothalamic forebrain regions (Section 1.4.2; Figure 1). However, despite pharmacological evidence that GLP-1 receptor signaling is critical for neuroendocrine and behavioral responses to cognitive stressors (Kinzig et al., 2003), no study has histologically demonstrated activation of GLP-1 neurons by cognitive stress.

In these cited studies and many others, including those in Chapters 2-4, 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. As a binary index of neural activation, the visible presence or absence of cFos immunolabeling in a given neuron poorly reflects the degree to which the neuron has been activated, and provides no information about that neuron's 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. As demonstrated above, the collective results of studies utilizing cFos indicate that GLP-1 and $PrRP^{+}$ A2 neurons are consistently activated by stimuli that present actual or anticipated threats to bodily homeostasis.

As reviewed above, hindbrain GLP-1 neurons and $PrRP^{+}$ A2 neurons receive broad sensory information regarding interoceptive state, and these populations are recruited by both visceral and cognitive stressors. Considering this, these neurons are optimally positioned to integrate information regarding metabolic state into neurally-mediated stress responses. Importantly, anatomical and functional evidence clearly demonstrate the capability of these

neurons to activate the HPA axis, elicit anxiety-like behavior, and produce stress-induced hypophagia.

1.6.2 PrRP$^+$ A2 & GLP-1 neurons activate the HPA axis

A2 NA and PrRP$^+$ neurons influence the HPA axis directly (Plotsky, 1987), as indicated by dense terminal arborizations within the mpPVN (Cunningham-Jr. and Sawchenko, 1988; Morales et al., 2000; Rinaman, 2010), asymmetric synapses between catecholaminergic terminals and CRH-positive neurons (Liposits et al., 1986b), and the presence of postsynaptic NA and PrRP receptors (Day et al., 1999; Roland et al., 1999). Functional evidence bolsters these anatomical findings, as NE increases excitatory postsynaptic potential frequency in the mpPVN, in part by enhancing glutamate signaling (Daftary et al., 2000), and the presence of NE or PrRP drives mpPVN cellular activity, stimulates CRH synthesis and release, and elevates plasma ACTH and CORT levels (Plotsky, 1987; Itoi et al., 1999; Helmreich et al., 2001; Cole and Sawchenko, 2002; Seal et al., 2002; Itoi et al., 2004; Mera et al., 2006). Furthermore, HPA axis activation is enhanced by co-administration of NE and PrRP (Maruyama et al., 2001), suggesting that these transmitters act in concert to increase hypothalamic outflow. Indeed, stimulation of A2 neurons (Day et al., 1985) or their ascending fiber tract (Plotsky, 1987) is sufficient to drive mpPVN activity and CRH secretion, while selective lesions of A2 NA projections to the mpPVN result in a substantial loss of neuroendocrine response to visceral stressors (Schiltz and Sawchenko, 2007; Bienkowski and Rinaman, 2008).

Hindbrain GLP-1 neurons are also poised to directly influence HPA axis activity, as these neurons densely innervate the mpPVN (Larsen et al., 1997b; Rinaman, 1999b, 2007; Tauchi et al., 2008) – which expresses GLP-1 receptors (Merchenthaler et al., 1999) – and synapse directly

onto CRH neurons (Sarkar et al., 2003a). GLP-1 signaling has strong excitatory effects on neural drive of the HPA axis, as *in vitro* recordings show that GLP-1 signaling increases the spike frequency of neurons in the PVN via facilitation of presynaptic glutamate release (Acuna-Goycolea and Pol, 2004). Furthermore, central administration of GLP-1 elicits cFos activation in mpPVN CRH neurons, subsequently elevating plasma ACTH and CORT (Larsen et al., 1997a; Kinzig et al., 2003). Recent findings from our lab indicate that GLP-1 soma (within the cNTS) and terminal arbors within the mpPVN co-localize vesicular glutamate transporter 2 (vGlut2) (Zheng & Rinaman, submitted), evidence that GLP-1 neurons are glutamatergic, and suggesting that activation of cNTS GLP-1 neurons can directly drive postsynaptic spiking via glutamate release. Together, this evidence strongly supports a role for $PrRP^{+}$ A2 neurons and hindbrain GLP-1 neurons in generating neuroendocrine stress responses.

1.6.3 NA/PrRP & GLP-1 signaling elicit anxiety-like behavior

Signaling from hindbrain GLP-1 neurons and $PrRP^{+}$ A2 neurons can elicit anxiety-like behavior via receptor activation within numerous midbrain and forebrain regions, including the ventrolateral (vl)BST and CeA, two highly interconnected nuclei responsible for the coordination of behavioral anxiety responses (Section 1.3.1). The vlBST gets particularly dense input from medullary NA and PrRP neurons (Forray et al., 2000; Banihashemi and Rinaman, 2006) (Figure 9), resulting in the highest concentrations of NE in the entire brain (Kilts and Anderson, 1986). Moreover, immobilization stress results in substantial increases in NE release within the vlBST, while pharmacological antagonism of NA receptors within this region decrease stress-induced anxiety-like behavior (Cecchi et al., 2002). Furthermore, selective lesion of NA innervation of

the vlBST (likely including $PrRP^+$ A2 neurons) prevents systemic yohimbine, an α2 adrenergic receptor antagonist, from increasing anxiety-like behavior (Zheng and Rinaman, 2013).

Both the CeA and vlBST receive axonal input from hindbrain GLP-1 neurons (Goke et al., 1995; Rinaman, 2010), providing a route through which GLP-1 neuronal activation can influence anxiety-like behavior. Indeed, anxiogenic behavioral outcomes can be attributed to forebrain GLP-1 signaling, since antagonism of central GLP-1 receptors decreases anxiety-like behavior, and GLP-1 injections directly into the CeA are sufficient to increase behavioral anxiety (Kinzig et al., 2003). Despite the presence of GLP-1 terminals within the vlBST (Rinaman, 2010), the contribution of vlBST GLP-1 signaling to anxiety-like behavior has not been determined. Together, these studies indicate that ascending projections from $PrRP^+$ A2 and GLP-1 neurons to the central extended amygdala contribute to anxiety-like behavior.

1.6.4 Signaling from hindbrain $PrRP^+$ A2 & GLP-1 neurons contribute to stress-induced hypophagia

A2 NA/$PrRP^+$ neurons are activated by stimuli that produce hypophagia, such as cognitive/visceral stressors (Section 1.6.1) and intake of a meal (Rinaman et al., 1998; Takayanagi et al., 2008). The strongest evidence that, once activated, these neurons play a causal role in stress-induced hypophagia comes from studies using a DβH-conjugated saporin toxin, which selectively lesions NA neurons. Selective lesions of A2 neurons (presumably including the $PrRP^+$ subset) result in reduction of visceral stress-induced hypophagia, and the degree to which food intake is suppressed is directly related to the degree of A2 neuron loss (Rinaman, 2003a; Rinaman and Dzmura, 2007). These studies, however, cannot differentiate the effects of functional loss of NE signaling from loss of PrRP signaling. Interestingly, DβH knockout mice –

which lack NE – continue to display hypophagic responses to visceral stress (Cannon and Palmiter, 2003). In these rodents, NA neurons are not lesioned and maintain the ability to synthesize and release PrRP, suggesting that PrRP signaling may be sufficient for stress-induced hypophagia. In support of this, central administration of PrRP reduces food intake (Lawrence et al., 2000; Lawrence et al., 2002), while knockout of PrRP (Takayanagi et al., 2008) or its receptor (Gu et al., 2004) results in hyperphagia. Lastly, varicose axons of NA and PrRP neurons can be found in the brainstem reticular formation (Yano et al., 2001), providing an anatomical route by which to influence meal size (Section 1.3.2)

GLP-1 neurons are also activated by stimuli that inhibit feeding (Rinaman, 1999b; Kreisler et al., 2014), and due to the development of selective and efficacious GLP-1 analogues and receptor antagonists, an abundance of pharmacological evidence supports the role of GLP-1 signaling in stress-induced hypophagia. First, lateral ventricular administration of GLP-1 suppresses food intake (Tang-Christensen et al., 1996; Turton et al., 1996; Rinaman, 1999a), an effect that is recapitulated with 4th ventricular GLP-1 administration in both intact and chronic supracollicular decerebrate rats (Kinzig et al., 2002; Hayes et al., 2008), indicating that GLP-1 signaling within the brainstem is sufficient to suppress food intake. While beneficial, pharmacological manipulations utilizing administration of agonist have limited physiological relevance, and much stronger evidence comes from studies blocking endogenous function using selective antagonists. When GLP-1 receptor antagonists are administered into the 4th ventricle or directly into the cNTS, rats indeed increase food intake (Hayes et al., 2009). Additionally, results from a study utilizing viral-mediated RNAi knockdown of GLP-1 in the NTS indicate that GLP-1 signaling is critical for regulation of food intake over a period of weeks (Barrera et al., 2011).

Together, these results show that GLP-1 signaling in the caudal brainstem is sufficient to suppress food intake.

The necessity of GLP-1 signaling for visceral stress-hypophagia has been demonstrated, as i.c.v. administration of GLP-1 receptor antagonist dose-dependently attenuates LiCl-induced hypophagia (Rinaman, 1999a; Seeley et al., 2000), and 4th i.c.v. antagonist administration diminishes suppression of food intake following peripheral LPS (Grill et al., 2004). As varicose axons of GLP-1 neurons are found in the reticular formation and other brainstem sites known to control oromotor outflow (e.g., the pontine parabrachial nucleus) (Rinaman, 2010; Llewellyn-Smith et al., 2011), it is likely that GLP-1 signaling suppresses food intake via modulation of pre-motor brainstem circuits. No studies, however, have assessed whether GLP-1 signaling is critical for hypophagic responses to cognitive stressors. Together, these findings indicate that PrRP^{+} A2 neurons and GLP-1 neurons play a critical role in visceral stress-induced hypophagia and are well positioned to mediate hypophagic responses to cognitive stressors as well.

1.7 HOW DOES FASTING ALTER CENTRALLY MEDIATED STRESS RESPONSES?

In response to actual or anticipated homeostatic stressors, the CNS elicits a constellation of neural, neuroendocrine, and behavioral stress responses that facilitate immediate survival and eventual restoration of homeostatic balance. A growing body of literature indicates that periods of caloric deficit attenuate many of these responses, including neural drive to the HPA axis, anxiety-like behavior, and stress-induced hypophagia. It is believed that these alterations represent an adaptive shift in stress responses during periods of negative energy balance, catering

stress responses to the animal's current metabolic state; however, the neural mechanisms by which this occurs remain unclear. Hindbrain GLP-1 and $PrRP^+$ A2 neurons are stress-sensitive and receive robust ingestive/metabolic information. Moreover, signaling from these populations of neurons contributes to each of the stress responses altered in the fasted state. Considering this, we proposed that the ability of caloric deficit to decrease stress hypophagia, central drive to the HPA axis, and anxiety-like behavior depends on reduced signaling from hindbrain GLP-1 and $PrRP^+$ A2 neurons. My dissertation project, comprised of the studies that follow, was designed to address various components of this overarching hypothesis.

In Chapter 2 we hypothesized that overnight food deprivation would attenuate the ability of supraphysiological doses of cholecystokinin-8 (CCK), a visceral stressor, to activate cFos within NA A2 neurons and hindbrain GLP-1 neurons. We further hypothesized that this would occur in conjunction with attenuation of neural activation in the mpPVN, indicative of reduced neural drive to the HPA axis. Our results indicate that, indeed, fasting substantially attenuated activation of brainstem A2/GLP-1 neurons and mildly reduced mpPVN activation.

Therefore, in Chapter 3 we hypothesized that cognitive stressors (i.e., restraint and elevated platform exposure) would activate GLP-1 and $PrRP^+$ A2 neurons in *ad libitum*-fed rats, and that fasting would attenuate this response. Furthermore, we proposed that fasting would reduce neural activation in the mpPVN and vlBST – forebrain regions implicated in HPA axis activation and behavioral anxiety, with a concomitant decrease in anxiety-like behavior. Finally, we utilized central pharmacological antagonism of GLP-1 receptors to empirically test the role of GLP-1 signaling in cognitive stress-induced hypophagia. We found that cognitive stressors robustly activated cFos within both GLP-1 and $PrRP^+$ A2 neurons, and this activation was nearly eliminated following an overnight fast. A similar reduction in stress-induced activation of the

vlBST was observed following fasting in conjunction with reduced anxiety-like behavior. Lastly, central antagonism of GLP-1 receptor signaling abolished cognitive stress-induced hypophagia.

Considering the marked ability of fasting to reduce both visceral and cognitive stress-induced activation of hindbrain neurons, we investigated the mechanisms by which metabolic state might attenuate activation of GLP-1 and $PrRP^+$ A2 neurons. Thus, in Chapter 4, we hypothesized that exogenous leptin administration and/or antagonism of ghrelin receptors in fasted rats would rescue the ability of systemic CCK to activate hindbrain GLP-1 and $PrRP^+$ A2 neurons. Our results indicate that ghrelin receptor antagonism – but not leptin administration – partially rescued activation of both GLP-1 and $PrRP^+$ A2 neurons in response to CCK.

Taken together, the results of my dissertation work demonstrate significant metabolic tuning of neural, behavioral, and neuroendocrine responses to visceral and cognitive stressors. It is not yet clear, however, whether fasting-mediated reductions in activation of brainstem neurons *cause* the attenuations observed in forebrain neural activation and anxiety-like behavior, leaving the door open for future investigation.

2.0 OVERNIGHT FOOD DEPRIVATION MARKEDLY ATTENUATES HINDBRAIN NORADRENERGIC, GLUCAGON-LIKE PEPTIDE-1, AND HYPOTHALAMIC NEURAL RESPONSES TO EXOGENOUS CHOLECYSTOKININ IN MALE RATS[1]

2.1 INTRODUCTION

It is well established that moderate to high systemic doses (i.e., > 3 μg/kg BW) of cholecystokinin-8 (CCK) suppress food intake (Gibbs et al., 1973; Gibbs and Smith, 1977) and activate hypothalamic endocrine neurons (Parrott et al., 1991; Verbalis et al., 1991; Katsuura et al., 1992; Chen et al., 1993). These behavioral and endocrine responses are critically dependent on CCK-A receptor-mediated activation of vagal sensory neurons that provide glutamatergic axonal input to the caudal nucleus of the solitary tract (NTS) (Smith et al., 1981; Raybould et al., 1985; Smith et al., 1985; Raybould et al., 1988; Moran et al., 1990; Kamilaris et al., 1992; Corp et al., 1993; Schwartz et al., 1994; Monnikes et al., 1997; Zittel et al., 1999; Sutton et al., 2004; Appleyard et al., 2007; Ritter, 2011). While neural circuits contained within the brainstem are

[1] The entirety of the work presented in Chapter 2 is from "Overnight food deprivation markedly attenuates hindbrain noradrenergic, glucagon-like peptide-1, and hypothalamic neural responses to exogenous cholecystokinin in male rats," by J.W. Maniscalco and L. Rinaman, 2013, *Physiology & Behavior*, *121*, p. 35-42. Copyright (2013) by Elsevier Inc. Reprinted with permission.

sufficient for the hypophagic effects of CCK (Grill and Smith, 1988), the ability of CCK to recruit hypothalamic endocrine neurons requires ascending projections from the NTS to the hypothalamus (Rinaman, 2003a).

A large variety of neurotransmitter molecules are expressed by neurons within the caudal NTS (Rinaman, 2011). Subsets of these NTS neurons directly modulate the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis via axonal projections to the medial parvocellular paraventricular nucleus of the hypothalamus (mpPVN) (Rinaman, 2007, 2011). Most of these projections arise from two phenotypically distinct populations of NTS neurons: noradrenergic (NA) neurons of the A2 cell group, and glucagon-like peptide-1 (GLP-1) neurons (Plotsky, 1987; Plotsky et al., 1989; Larsen et al., 1997b; Larsen et al., 1997a; Kinzig et al., 2003; Bienkowski and Rinaman, 2008). A2 and GLP-1 neurons densely innervate the mpPVN (Cunningham-Jr. and Sawchenko, 1988; Larsen et al., 1997b; Rinaman, 1999b; Tauchi et al., 2008), synapsing directly onto corticotropin-releasing hormone (CRH)-positive neurons at the apex of the HPA axis (Liposits et al., 1986b; Sarkar et al., 2003a). Furthermore, intraventricular and local parenchymal injections of either GLP-1 or norepinephrine (NE) induce cFos expression within the mpPVN and increase plasma levels of corticosterone (CORT) (Larsen et al., 1997a; Cole and Sawchenko, 2002; Kinzig et al., 2003). Accumulated evidence suggests that CCK-induced activation of A2 and GLP-1 neurons underlies the ability of CCK to activate the HPA axis and generate other neuroendocrine responses (Kendrick et al., 1991; Verbalis et al., 1991; Rinaman et al., 1995; Verbalis et al., 1995; Rinaman, 1999b; Babic et al., 2009). Several published studies have reported dose-response relationships for the ability of systemic CCK to inhibit food intake (Gibbs et al., 1973; Moran et al., 1997), increase NTS cFos expression (Monnikes et al., 1997; Zittel et al., 1999; Babic et al., 2009), and activate the HPA axis (Verbalis et al., 1991; Kamilaris

et al., 1992). However, none of these reports included phenotypic identification of the NTS neurons activated by CCK, and none has assessed the degree to which NTS cFos expression is associated with cFos activation within the mpPVN.

Interestingly, food deprivation attenuates the ability of systemic CCK to suppress food intake (Stein et al., 1986; McMinn et al., 2000), perhaps due to reduced circulating levels of leptin in food-deprived rats (Dallman et al., 1999; Mastronardi et al., 2000; Pico et al., 2002). Leptin potentiates the excitatory effects of exogenous CCK on vagal sensory afferents (Peters et al., 2006c; Peters et al., 2006a) and NTS neurons (Wang et al., 1998; Emond et al., 1999; Wang et al., 2000), including A2 neurons (Williams et al., 2008), and the ability of food deprivation to attenuate CCK-induced hypophagia is abolished by systemic leptin administration (McMinn et al., 2000). While it is clear that food deprivation reduces the hypophagic potency of CCK, the ability of CCK to activate phenotypically-identified A2 and GLP-1 neurons in rats after food deprivation has not been assessed, and there are no published reports regarding the effect of food deprivation on CCK-induced activation of the HPA axis. Considering this, the present study was designed to test three hypotheses: first, that increasing doses of CCK activate increasing numbers of A2, GLP-1, and mpPVN neurons to express cFos; second, that A2, GLP-1, and mpPVN neuronal activation occurs in a correlated manner; and third, that food deprivation significantly attenuates the ability of CCK to increase cFos expression within these populations of hindbrain and hypothalamic neurons.

2.2 METHODS

Animals

Adult male Sprague-Dawley rats (Harlan, IN; 225-275g BW; n = 51) were housed singly in hanging stainless steel wire mesh cages in a temperature-controlled room (20-22°C) with a 12/12 hr light/dark cycle (lights on at 0700 hr). Rats had *ad libitum* access to pelleted chow (Purina 5001) and water, except as noted. All 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.

Injections and perfusion

Rats were weighed one day before CCK administration to determine proper dosage. Rats were returned to their home cage with *ad libitum* chow access (ad lib; n = 28), or were deprived of food (but not water) for 16-18 hours overnight in their home cage (DEP; n = 23) before CCK or vehicle treatment. On the day of the experiment rats from each group were removed from their home cage between 0830 and 1030 hr and injected intraperitoneally (i.p.) with 1.0 ml of sterile 0.15M NaCl containing sulfated CCK-8 (Bachem; H-2080) as follows: 0 μg/kg BW (n = 9 ad lib; n = 9 DEP); 3 μg/kg BW (n = 7 ad lib; n = 4 DEP); 10 μg/kg BW (n = 7 ad lib; n = 4 DEP). These CCK doses significantly inhibit food intake in rats (McCann et al., 1989; McMinn et al., 2000; Rinaman, 2003a), stimulate pituitary hormone secretion (McCann et al., 1989; Kamilaris et al., 1992), and activate cFos expression by CRH-positive and other PVN neurons (Verbalis et al., 1991; Day et al., 1994; Noetzel et al., 2009). CCK was dissolved in vehicle just before injection, and rats were returned to their home cage immediately after injection. Additional nonhandled (NH) control rats (n = 5 ad lib; n = 6 DEP) received no i.p. injection and remained

undisturbed in their home cages until perfusion within the same time period. Ninety minutes after i.p. injection, rats were deeply anesthetized with pentobarbital sodium (39 mg/1.0 ml i.p., Fatal Plus Solution; Butler Schein) and perfused transcardially with a brief saline rinse followed by fixative (100 ml of 2% paraformaldehyde and 1.5% acrolein in 0.1M phosphate buffer, followed by 100 ml of 2% paraformaldehyde alone) (McLean and Nakane, 1974). Brains were post-fixed *in situ* overnight at 4°C, then removed from the skull and cryoprotected for 24-48 hr in 20% sucrose. Brains were blocked and sectioned coronally (35μm) on a freezing stage microtome. Sections were collected in six serial sets, and stored at -20°C in cryopreservant solution (Watson et al., 1986) until immunocytochemical processing.

Immunohistochemistry

Primary and secondary antisera were diluted in 0.1M phosphate buffer containing 0.3% Triton X-100 and 1% normal donkey serum. One set of tissue sections from each rat was incubated in a rabbit polyclonal antiserum against cFos (1:50,000; kindly provided by Dr. Philip Larsen, Denmark), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch). Sections were treated with Elite Vectastain ABC reagents (Vector) and reacted with diaminobenzidine (DAB) intensified with nickel sulfate to produce a blue-black nuclear cFos reaction product. To identify NA neurons of the A2 cell group, cFos-labeled sections were subsequently processed to localize dopamine-β-hydroxylase (DβH) using mouse anti-DβH (1:50,000; Millipore, MAB308) followed by biotinylated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch). Sections were reacted using Elite Vectastain ABC reagents and plain DAB to produce a brown cytoplasmic reaction product.

A second set of sections from each rat was incubated in a rabbit polyclonal antiserum against GLP-1 (1:10,000; Bachem, T-4363), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch), Elite Vectastain ABC reagents, and plain DAB to produce a brown cytoplasmic reaction product. These sections then were processed for immunofluorescent localization of cFos using a higher primary antibody concentration (1:5,000) followed by incubation in Cy3-conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch) to produce a red fluorescent nuclear signal.

Quantification of cFos expression by NTS A2 neurons

Hindbrain tissue sections were analyzed using a light microscope and a 40X objective to determine the number of double-labeled, DβH/cFos-positive neurons within the NTS. Double-labeled neurons were counted bilaterally through the rostrocaudal extent of the A2 cell group (i.e., from the cervical spinal cord through the NTS just rostral to the area postrema; ~15.46 mm to 13.15 mm caudal to bregma), and then averaged as counts per section. Criteria for counting a neuron as double-labeled included brown DβH cytoplasmic labeling and a nucleus that contained visible blue-black cFos immunolabeling, regardless of intensity.

Quantification of cFos expression by GLP-1 neurons

As there is no current evidence that GLP-1 neurons residing in the lateral reticular formation are anatomically or functionally distinct from their counterparts residing in the NTS, all visible GLP-1 neurons were imaged in the NTS and adjacent reticular formation using a 20X objective on an Olympus microscope equipped for brightfield and epifluorescence, and photographed using a digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Neurons were counted in

photographic images using Adobe Photoshop CS4 image software. Criteria for counting a neuron as GLP-1-positive included brown cytoplasmic labeling and a visible nucleus. Neurons were considered cFos positive if their nucleus contained red fluorescent cFos immunolabeling, regardless of intensity. Counts of GLP-1 neurons were performed throughout the rostrocaudal extent of the GLP-1 cell group (i.e., from the cervical spinal cord through the NTS just rostral to the area postrema; ~15.46 mm to 13.15 mm caudal to bregma), and then averaged as counts per section.

Quantification of cFos expression in the mpPVN

cFos/GLP-1-labeled sections were viewed on the Olympus photomicroscope described above. Using a 10X objective, photographic images were captured from a single selected rostrocaudal level of the mpPVN (approximately 1.78 mm caudal to bregma). This selected level was characterized by dense GLP-1 terminal labeling that clearly defined the boundaries of the mpPVN (see Figure 4). cFos-positive neurons within this defined region of the mpPVN were counted bilaterally on images using Adobe Photoshop CS4 image software. The criterion for counting a neuron as cFos-positive was the presence of visible red fluorescent nuclear immunolabeling, regardless of intensity.

Statistics

Two-way multivariate ANOVA was used to reveal main effects of, or interactions between, CCK injection group (NH, 0, 3, & 10 μg/kg BW) and feeding status (ad lib vs. DEP) on cFos counts within the hindbrain and hypothalamus. When F-values indicated significant interactions or main effects, the ANOVA was followed by post hoc tests with Bonferroni correction for

multiple comparisons. Differences were considered significant when $p < 0.05$. Pearson's R correlation coefficient was used to determine whether significant correlations existed between treatment-induced activation of A2 neurons, GLP-1 neurons, and mpPVN neurons. Outlier tests identified 4 animals with data that lay more than 2 standard deviations from the group mean for a given dependent variable. Data from these animals were excluded from all analyses and do not contribute to the results.

2.3 RESULTS

Two-way ANOVA revealed significant main effects of CCK injection group and feeding status on cFos expression in DβH- and GLP-1-positive hindbrain neurons, and on cFos expression within the mpPVN. There also were significant interactions between injection group and feeding status on cFos activation in A2 and GLP-1 neurons, but not on mpPVN cFos activation (Table 1).

Table 1. Multivariate ANOVA Statistics

Multivariate ANOVA main effects, interactions, and *p*-values for cFos counts in ad lib-fed or DEP rats following NH, 0 µg/kg, 3 µg/kg, and 10 µg/kg CCK.

Effect Source	Double-Label Count	F	*p* value
CCK Injection [*F*(3,41)]	Number of cFos/DβH Neurons – NTS Total	87.18	$p < .00$*
NH vs. 0 µg/kg vs. 3 µg/kg vs. 10 µg/kg	Number of cFos/GLP-1 Neurons	5.52	$p < .00$*
	Number of cFos Neurons in mpPVN	108.92	$p < .00$*
Feeding Status [*F*(1,41)]	Number of cFos/DβH Neurons – NTS Total	35.77	$p < .00$*
Ad lib vs. DEP	Number of cFos/GLP-1 Neurons	59.16	$p < .00$*
	Number of cFos Neurons in mpPVN	16.08	$p < .00$*
Interaction [*F*(3,41)]	Number of cFos/DβH Neurons – NTS Total	5.15	$p < .00$*
CCK Injection X Feeding Status	Number of cFos/GLP-1 Neurons	3.26	$p = .03$*
	Number of cFos Neurons in mpPVN	0.58	$p = .63$

*statistically significant

A2 neuronal activation

CCK increased cFos expression within the caudal NTS (Figure 2A), consistent with previous reports (Monnikes et al., 1997; Zittel et al., 1999; Babic et al., 2009). Our new findings indicate that this includes a dose-dependent recruitment of DβH-positive A2 neurons (Figure 2B). Very few A2 neurons expressed cFos in NH control rats, regardless of feeding status. A2 activation was significantly increased in ad-lib fed rats, but not in DEP rats, after i.p. injection of saline vehicle (0 μg/kg CCK). CCK delivered at doses of 3 or 10 μg/kg further increased cFos expression by A2 neurons in both feeding status groups, but these effects were significantly attenuated in DEP rats (Figure 2B).

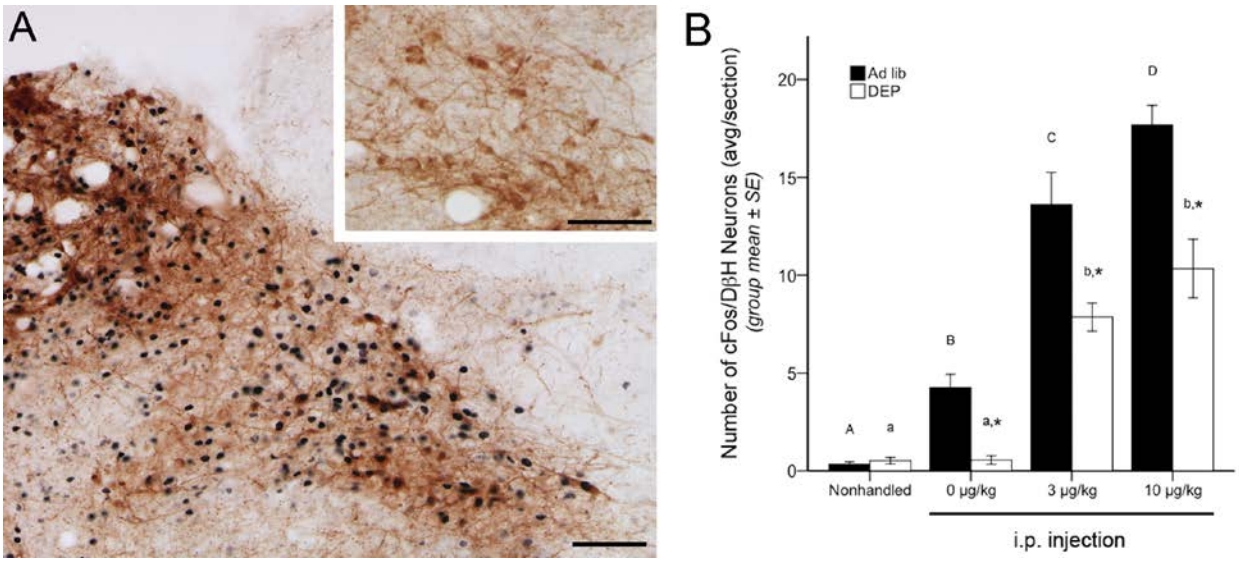

Figure 2. Overnight fasting attenuates CCK-induced cFos activation of A2 neurons

Representative color images and summary data for neuronal cFos expression (black nuclear label) within the A2 region of the caudal NTS. A, NTS cFos activation at the rostrocaudal level of the area postrema, approximately 14.16 mm caudal to bregma. In the larger image, robust cFos expression is present in a rat after CCK (10 µg/kg BW), including activation of DβH-positive (brown) neurons comprising the A2 cell group. *Inset*, little or no cFos activation is present in an ad lib-fed rat after i.p. vehicle (0 µg/kg BW CCK). B, bar graph illustrating the number of double labeled (i.e., both cFos- and DβH-positive) NTS neurons in ad lib-fed rats (solid bars) or food-deprived rats (DEP; open bars) after no i.p. injection (nonhandled) or after injection of CCK at doses of 0 (vehicle), 3, or 10 µg/kg BW. See Table 1 for two-way ANOVA results. In both feeding status groups, CCK dose-dependently increased cFos expression in DβH-positive A2 neurons, but cFos activation was significantly attenuated in food-deprived rats after vehicle or CCK injections. Asterisks over DEP bars indicate significant differences ($p < .05$) in the number of double-labeled NTS neurons compared to ad lib-fed rats in the same i.p. treatment group. Within the same feeding status group (i.e., ad lib or DEP), bars with different letters are significantly different ($p < .05$). Scale bars in A = 100 µm.

GLP-1 neuronal activation

In contrast to the cFos activation profile for DβH-positive A2 neurons, GLP-1 neurons did not respond to CCK in a dose-dependent manner (Figure 3). In ad lib-fed rats, GLP-1 cFos activation was robustly increased by every i.p. treatment, with no additional effect of CCK (Figure 3C). In DEP rats, cFos expression by GLP-1 neurons was virtually abolished in NH controls and after i.p. injection of vehicle or 3 μg/kg. DEP rats displayed a trend towards increased GLP-1 activation after the 10 μg/kg dose of CCK (Figure 3C), but this did not reach significance.

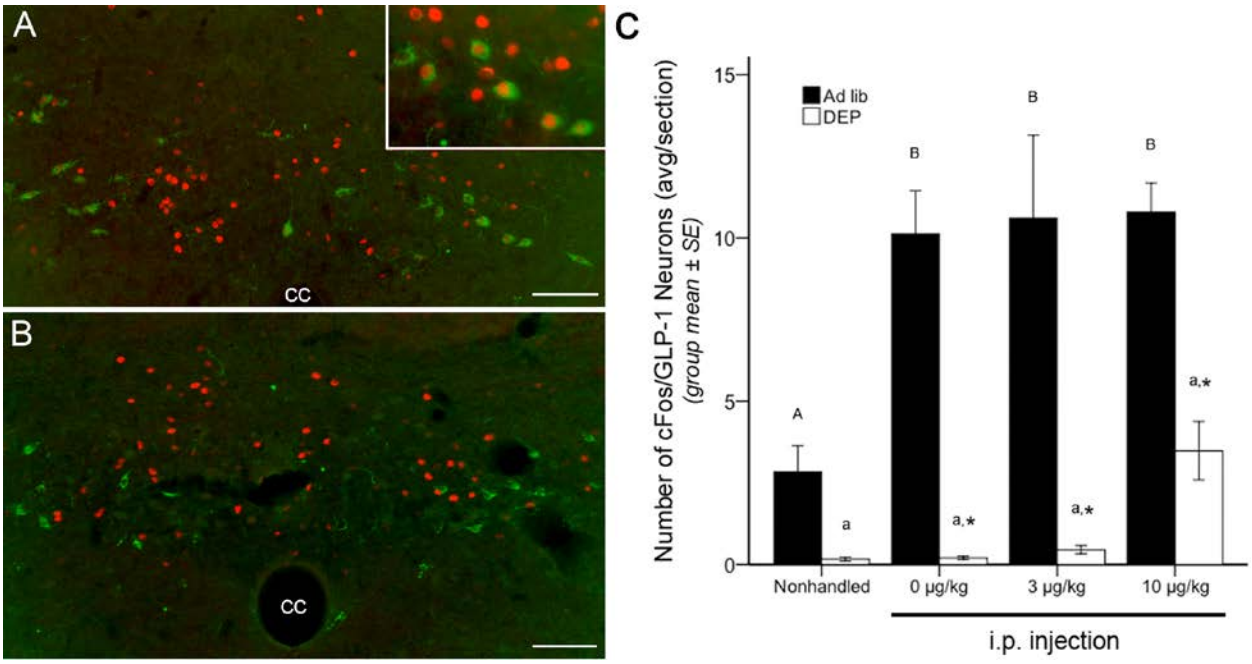

Figure 3. Overnight fasting nearly eliminates CCK-induced cFos activation of hindbrain GLP-1 neurons

Representative color images and summary data for neuronal cFos expression (red fluorescence) within GLP-1-positive neurons (green fluorescence) of the caudal NTS. A, in an ad lib-fed rat, many GLP-1 neurons express cFos after i.p. injection of CCK (3 µg/kg BW). *Inset*, higher magnification view of several double-labeled neurons. B, in a food-deprived rat, GLP-1 neurons are not activated to express cFos after CCK (3 µg/kg BW), despite activation of other caudal NTS neurons. C, bar graph illustrating the number of double labeled (i.e., both cFos- and GLP-1-positive) neurons within the NTS and adjacent reticular formation in ad lib-fed rats (solid bars) or food-deprived rats (DEP; open bars) after no i.p. injection (nonhandled) or after injection of CCK at doses of 0 (vehicle), 3, or 10 µg/kg BW. See Table 1 for two-way ANOVA results. In ad lib-fed rats, a moderate number of GLP-1 neurons express cFos in the nonhandled control condition, and this number more than doubled after i.p. injection of vehicle or CCK, regardless of dose. Food deprivation markedly suppressed cFos expression by GLP-1 neurons, with a non-significant trend towards increased activation seen after the highest CCK dose (10 µg/kg BW). Asterisks indicate significantly reduced ($p < .05$) GLP-1 cFos expression in DEP rats compared to GLP-1 cFos expression in ad lib-fed rats in the same i.p. treatment group. Within the same feeding status group (i.e., ad lib or DEP), bar values with different letters are significantly different ($p < .05$). cc, central canal. Scale bars in A and B = 100 µm.

mpPVN neuronal activation

The mpPVN displayed relatively low levels of cFos labeling in both ad lib-fed and DEP rats in the NH and vehicle-injected control groups (Figure 4A,C), and cFos was markedly increased in rats from both feeding status groups after 3 or 10 μg/kg of CCK (Figure 4B,C). DEP rats, however, displayed less mpPVN cFos labeling overall compared to labeling in ad lib-fed rats (see Table 1).

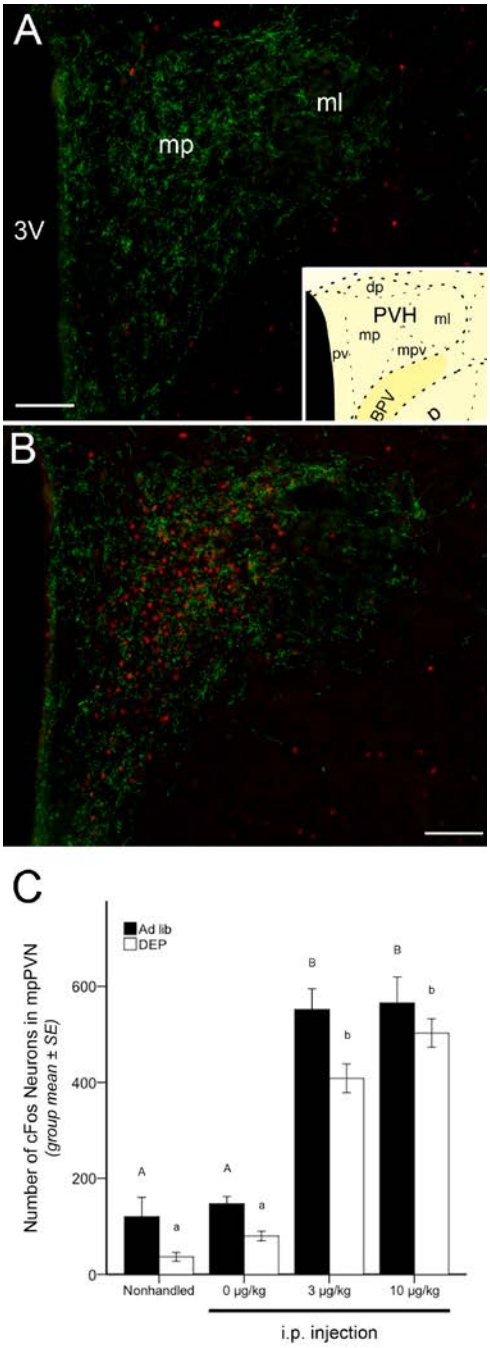

Figure 4. Overnight fasting modestly attenuates mpPVN cFos activation

Representative color images and summary data for neuronal cFos expression (red fluorescence) within the GLP-1 (green fluorescence) terminal-rich region of the mpPVN. A, little cFos is present in the mpPVN of an ad lib-fed, nonhandled control rat. B, robust mpPVN cFos activation is present in an ad lib-fed rat injected with CCK (10 µg/kg BW). mpPVN images were captured and cFos quantified in sections approximately 1.78 mm caudal to bregma, as

schematized in the inset in panel A (Swanson, 2004). C, bar graph illustrating the number of cFos-positive mpPVN neurons in ad lib-fed rats (solid bars) or food-deprived rats (DEP; open bars) after no i.p. injection (nonhandled) or after injection of CCK at doses of 0 (vehicle), 3, or 10 μg/kg BW. See Table 1 for two-way ANOVA results. In both feeding status groups, low levels of mpPVN cFos activation were present in nonhandled or vehicle-injected rats, and CCK delivered at doses of 3 and 10 μg/kg produced marked activation of mpPVN cFos. Food-deprived rats displayed less mpPVN cFos labeling overall compared to labeling in ad lib-fed rats. Within the same feeding status group (i.e., ad lib or DEP), bars with different letters indicate significant i.p. treatment-induced differences ($p < .05$) in mpPVN cFos activation. mp = medial parvocellular subdivision of the PVN; ml = lateral magnocellular subdivision of the PVN; 3V = third ventricle. Scale bars in A and B = 100 μm.

Correlational analyses

Activation of NTS DβH-positive A2 neurons correlated strongly with activation of mpPVN neurons (Figure 5A). This positive correlation existed in both ad lib-fed and DEP rats when data from these groups were analyzed independently (see Table 2 for a breakdown of Pearson correlation values and significance by injection and feeding status group). The number of cFos-positive GLP-1 neurons also significantly correlated with cFos expression in the mpPVN (Figure 5B), although the GLP-1/mpPVN cFos correlation was less robust than the DβH/mpPVN cFos correlation (Figure 5A). The GLP-1/mpPVN correlation also was significant in both ad lib-fed and DEP animals when these groups were analyzed independently (Table 2). In addition, the number of cFos-positive GLP-1 neurons correlated positively with cFos expression in DβH neurons (Figure 5C), and this GLP-1/DβH cFos correlation also remained significant when the ad lib-fed and DEP groups were analyzed independently (Table 2).

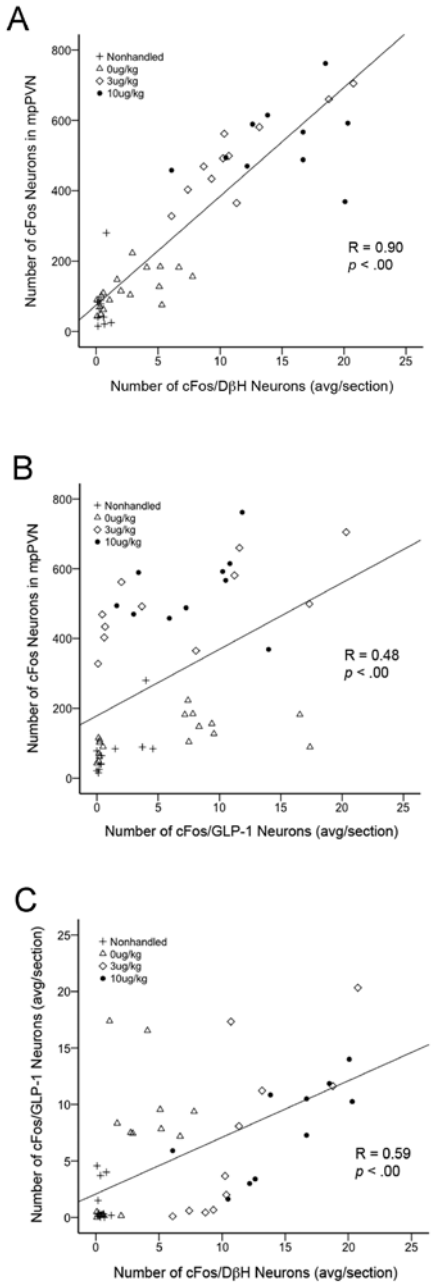

Figure 5. A2 & GLP-1 cFos activation correlate strongly with mpPVN cFos activation

Scatter plots depicting significant positive correlations between treatment-induced activation of (A) mpPVN and NTS DβH neurons, (B) mpPVN and GLP-1 neurons, and (C) GLP-1 and DβH neurons. In each plot, individual symbols represent data from a single animal. Different symbol styles represent different i.p. treatment groups, as indicated in the key. See Table 2 for a breakdown of correlation R values and significance by i.p. treatment and feeding status groups.

Table 2. Correlations in cFos expression between NTS DβH neurons, brainstem GLP-1 neurons, and mpPVN neurons.

			DβH/mpPVN		GLP-1/mpPVN		DβH/GLP-1	
		n	R value	*p* value	R value	*p* value	R value	*p* value
Ad Lib	Nonhandled	5	0.93	.02*	0.44	.46	0.29	.64
	0 µg/kg CCK	9	0.22	.55	-0.35	.36	-0.34	.37
	3 µg/kg CCK	7	0.81	.03*	0.45	.31	0.64	.12
	10 µg/kg CCK	6	-0.22	.67	-0.11	.83	0.40	.44
	Total	27	0.88	< .00*	0.38	.05*	0.48	.01*
DEP	Nonhandled	6	-0.22	.68	-0.15	.77	-0.09	.87
	0 µg/kg CCK	8	0.57	.14	0.44	.27	-0.20	.64
	3 µg/kg CCK	4	0.90	.10	0.71	.29	0.79	.21
	10 µg/kg CCK	4	0.61	.39	-0.26	.74	-0.73	.27
	Total	22	0.97	< .00*	0.67	< .00*	0.60	< .00*

*statistically significant

[a] Pearson correlation values and significance for relationships between cFos expression in NTS DβH neurons and mpPVN neurons (DβH/mpPVN), between brainstem GLP-1 neurons and mpPVN neurons (GLP-1/mpPVN), and between NTS DβH neurons and brainstem GLP-1 neurons (DβH/GLP-1).

2.4 DISCUSSION

Results from previous studies indicate that CCK elicits neuroendocrine responses via vagally-mediated recruitment of NTS neurons, including NA neurons of the A2 cell group, that project to the hypothalamus (Rinaman et al., 1995; Monnikes et al., 1997; Rinaman, 2003a). Hindbrain GLP-1 neurons are also known to be activated in rats receiving a very high (i.e., 100 μg/kg BW) dose of CCK (Rinaman, 1999b), and GLP-1 axonal projections target the mpPVN (Larsen et al., 1997b; Tauchi et al., 2008; Rinaman, 2010), suggesting an additional role for GLP-1 signaling in CCK-induced stimulation of the HPA axis. Although we did not measure stress hormone release in the present study, CCK at doses lower, higher, and similar to those used here stimulates pituitary hormone secretion (McCann et al., 1989; Kamilaris et al., 1992) and activates cFos expression by CRH-positive and other PVN neurons in rats (Verbalis et al., 1991; Day et al., 1994; Noetzel et al., 2009). However, the degree to which lower doses of CCK recruit phenotypically identified A2 and GLP-1 neurons, and the specific relationship between activation of these brainstem neurons and those in the mpPVN, has not been examined. Results from the present study reveal a CCK dose-dependent increase in A2 neuronal activation that strongly correlates with activation of mpPVN neurons. Conversely, i.p. injection by itself robustly increased the number of GLP-1 neurons expressing cFos, with no additional effect of CCK. Overnight food deprivation nearly abolished cFos expression in GLP-1 neurons, significantly attenuated CCK-induced cFos expression in A2 neurons, and had an overall effect to reduce cFos expression within the mpPVN.

Our results point to heterogeneity between A2 and GLP-1 neurons in their sensitivity to i.p. injection stress and CCK, although both hindbrain neuronal populations are clearly sensitive to changes in feeding status, as are neurons within the mpPVN. We propose that the apparent metabolic sensitivity of A2 and GLP-1 neurons contributes to the overall suppressive effect of food deprivation on mpPVN cFos expression. Since i.p. injection and exogenous CCK are capable of recruiting stress-sensitive hindbrain and hypothalamic neurons, the robust effect of an overnight fast to dampen treatment-induced cFos expression suggests that central neuronal responses to other stressful stimuli may also be modulated by feeding status. Indeed, fasting has been reported to interfere with the ability of animals to mount an HPA axis response to psychological stress (De Boer et al., 1989; Rohleder and Kirschbaum, 2007).

A2 neurons

A2 neurons rarely expressed cFos in non-manipulated, ad lib-fed rats, but i.p. vehicle and CCK dose-dependently activated this neuronal population. These new findings extend previous reports that CCK dose-dependently increases global NTS cFos expression (Monnikes et al., 1997; Zittel et al., 1999). Interestingly, while food deprivation attenuated A2 cFos expression across all i.p. injection groups, deprivation did not abolish CCK dose-dependent increases in A2 cFos activation. Thus, while A2 neurons are sensitive to feeding status, this neuronal population retains CCK sensitivity in food-deprived rats. Since rats in the present study were not acclimated to handling or i.p. injection before experimental treatment, it is possible that fasting attenuated A2 neural sensitivity to the stress of the injection procedure without altering sensitivity to CCK, or *vice versa*. Additionally, injection stress may interact non-additively with CCK to elicit A2

cFos activation, obfuscating the precise contribution of each stimulus to A2 cFos recruitment in non-acclimated rats. It will be important to empirically assess these possibilities in future studies.

The strong correlation between CCK-induced activation of A2 and mpPVN neurons suggests, but does not demonstrate, a causal relationship between these variables. Additional evidence supporting this suggestion comes from studies in which loss or interruption of NA projections from the NTS to the PVN markedly reduces the ability of visceral stimuli – including CCK – to recruit the HPA stress axis (Rinaman, 2003a; Ritter et al., 2003; Rinaman and Dzmura, 2007; Bienkowski and Rinaman, 2008). A2 neurons are also necessary for exogenous CCK to reduce food intake in rats (Rinaman, 2003a). Thus, our finding that fasting attenuates treatment-induced A2 neuronal activation may reveal a mechanism through which feeding status modulates both behavioral (e.g., hypophagic) and neuroendocrine responses to CCK and other visceral stimuli. Importantly, however, NTS inputs to the hypothalamus – and thus HPA axis activation – are unnecessary for CCK-induced hypophagia, as chronic decerebrate and neonatal rats (both of which lack functional connectivity between the NTS and PVN) display hypophagia after CCK treatment (Grill and Smith, 1988; Robinson et al., 1988; Rinaman et al., 1994). Moreover, it is likely that the A2 neurons responsible for brainstem-mediated hypophagic responses are distinct from those that mediate HPA axis responses to exogenous CCK, as toxin-induced loss of PVN-projecting A2 neurons does not alter CCK-induced hypophagia (Ritter et al., 2001). We interpret these collective results as evidence that exogenous CCK recruits A2 neurons that inhibit food intake via local brainstem projections, while also recruiting A2 neurons that project to the hypothalamus to elicit neuroendocrine activation.

GLP-1 neurons

Hindbrain GLP-1 neurons displayed moderate baseline levels of cFos expression (i.e., ~2-3 activated neurons/section, corresponding to ~20% of all GLP-1 neurons) in non-manipulated, ad lib-fed rats. In contrast to the A2 population, GLP-1 neurons were markedly and similarly activated to express cFos in ad lib-fed rats subjected to any i.p. injection, regardless of CCK content. Approximately 10 GLP-1 neurons per section (i.e., ~60% of all GLP-1 neurons) were activated in each i.p. treatment group. Thus, GLP-1 neurons appear to be uniquely sensitive to the mild stress of handling and/or i.p. injection, which might have masked any additional activation by CCK.

Surprisingly, GLP-1 neural cFos expression was nearly abolished in rats after overnight food deprivation, evidence that the baseline cFos expression and robust response to i.p. injection displayed by GLP-1 neurons is strongly modulated by feeding status. Despite an apparent trend towards increased GLP-1 cFos activation in food-deprived rats after the 10 μg/kg dose of CCK, this increase did not reach significance. A higher CCK dose (e.g., 50-100 μg/kg) might be sufficient to overcome the dampening effect of food deprivation on GLP-1 cFos activation, but this remains to be determined. Although GLP-1 and mpPVN cFos expression was positively correlated, the correlation was much stronger for A2 and mpPVN cFos expression, suggesting that GLP-1 neurons have less direct effects on mpPVN responses to CCK. Consistent with this, previous results from our laboratory indicate that in the absence of normal A2 neuronal signaling, the ability of exogenous CCK to activate hindbrain GLP-1 neurons is preserved, but is insufficient to support either CCK-induced hypophagia or PVN cFos expression (Rinaman, 2003a). Nevertheless, central GLP-1 receptor signaling has been implicated in HPA stress axis and anxiogenic responses to visceral stress (Kinzig et al., 2003), perhaps through gating the post-

synaptic effects of another transmitter molecule (Acuna-Goycolea and Pol, 2004). Thus, the observed attenuation of GLP-1 cFos activation following deprivation suggests that reduced GLP-1 signaling might contribute to the blunted neuroendocrine stress responses that have been previously reported in food-deprived rats (De Boer et al., 1989; Rohleder and Kirschbaum, 2007).

mpPVN neurons

Considering the well-documented excitatory effects of NA and GLP-1 signaling on mpPVN neural activity (Day et al., 1985; Daftary et al., 2000; Cole and Sawchenko, 2002; Rinaman, 2007) and the markedly blunted A2 and GLP-1 cFos activation in food-deprived rats, it is surprising that food deprivation generated only a mild overall attenuation of mpPVN cFos expression under all treatment conditions. In both ad lib-fed and food-deprived rats, mpPVN cFos activation occurred in a step-wise manner, with comparable levels of cFos observed in NH and i.p. vehicle-injected control groups, and substantially increased cFos after CCK administered at either the 3 or 10 μg/kg dose. It is possible that the comparable magnitude of mpPVN cFos activation after both CCK doses is due to a ceiling effect, as a previous study reported that doses of 5 and 10 μg/kg CCK elicited similar increases in plasma adrenocorticotropic hormone (ACTH) (Kamilaris et al., 1992). The similar number of cFos-positive mpPVN neurons after both CCK doses may also reflect an inherent limitation in interpreting cFos data, as the visible presence or absence of cFos protein in a given neuron is binary, poorly reflecting the degree to which the neuron has been activated, and providing no information about that neuron's spike frequency or amplitude. Furthermore, we do not know the extent to which hypophysiotropic CRH-positive mpPVN neurons were activated after each CCK dose. Nevertheless, the overall

blunted mpPVN cFos expression observed in food-deprived rats in the present report is consistent with prior evidence that fasting blunts ACTH responses to stress (De Boer et al., 1989; Rohleder and Kirschbaum, 2007), which may result from reduced excitatory drive to the mpPVN from hindbrain A2 and GLP-1 neurons.

Potential signals of feeding status

Attenuation of A2, GLP-1, and mpPVN neural cFos expression in food-deprived rats could result from loss of an excitatory signal that is present in ad lib-fed rats, generation of an inhibitory signal associated with food deprivation, or both. Fasting produces a multitude of metabolic and physical changes (Dallman et al., 1999), with reductions in circulating leptin, increases in circulating ghrelin, and reduced gastric distention standing out as potential candidate effectors of deprivation-induced changes in central neural activity. Leptin levels fall in rats after overnight food deprivation (Dallman et al., 1999; Pico et al., 2002), and leptin enhances CCK signaling at the level of the vagus nerve (Peters et al., 2004; Peters et al., 2006b), the NTS (Wang et al., 1998; Emond et al., 1999) (particularly A2 neurons (Williams et al., 2008)), and the PVN (Wang et al., 1998; Emond et al., 1999). Reduced gastric distention signals in food deprived rats might also attenuate central neural cFos responses to CCK, since CCK and gastric distention act synergistically to activate vagal afferent inputs (Schwartz et al., 1991b; Schwartz et al., 1993) that modulate NTS neuronal activity (Raybould et al., 1988). It is less likely that increased ghrelin signaling after fasting (Toshinai et al., 2001) is responsible for attenuated central neural responses to CCK, since i.p. ghrelin does not suppress the ability of CCK to activate cFos expression in the NTS or PVN (Kobelt et al., 2005).

Conclusions

Our results show that CCK- and i.p. injection-induced cFos activation of A2, GLP-1, and mpPVN neurons is strongly modulated by short-term fasting. As these neural populations are activated following a diverse array of stressors (Li et al., 1996; Rinaman, 1999b), including "cognitive-type" restraint stress (Dayas et al., 2001b; Maniscalco et al., 2012), we expect that food deprivation would also alter hindbrain and hypothalamic neural responses to restraint and other stressors. Considering that A2, GLP-1, and mpPVN neurons play important roles in generating behavioral and physiological responses to stress, feeding status may impact an animal's ability to mount these responses by altering the sensitivity of hindbrain and hypothalamic neurons to their afferent inputs. It will be critical for future studies to identify the mechanisms by which food deprivation alters neural activation in these brainstem and hypothalamic populations, and to assess the functional consequences of altered neuronal signaling.

3.0 OVERNIGHT FASTING REVEALS A LINK BETWEEN HINDBRAIN GLUCAGON-LIKE PEPTIDE-1 AND PROLACTIN-RELEASING PEPTIDE/NORADRENERGIC NEURONS AND BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO COGNITIVE STRESS

3.1 INTRODUCTION

Health and well-being depend on appropriate physiological and behavioral responses to perceived and actual homeostatic threats (i.e., stressors) (Chrousos, 1998). These responses include activation of the hypothalamic-pituitary-adrenal (HPA) axis, which occurs following recruitment of corticotropin-releasing hormone (CRH) neurons in the medial parvocellular paraventricular hypothalamus (mpPVN) (Ulrich-Lai and Herman, 2009). CRH neural activation promotes systemic release of adrenocorticotropic hormone (ACTH) to drive glucocorticoid (GC) synthesis in the adrenal cortex, GCs then elicit a multitude of adaptive physiological, neural, and behavioral changes (Sapolsky et al., 2000; Charmandari et al., 2005). Stress-induced activation of the HPA axis often occurs in concert with affective anxiety and suppression of food intake (i.e., hypophagia), producing a state characterized by behavioral inhibition, hyper-vigilance, and avoidance responses (Davis, 2002; Davis et al., 2010; Calvez et al., 2011; Maniam and Morris, 2012).

Acute or chronic periods of caloric deficit attenuate anxiety-like behavior (Genn et al., 2003; Inoue et al., 2004) and central drive to the HPA axis, as evidenced by decreased mpPVN CRH mRNA expression (Brady et al., 1990; Kiss et al., 1994), decreased baseline mpPVN cFos activation (Dallman et al., 1999), and decreased plasma ACTH concentrations at baseline and in response to restraint stress (Hanson et al., 1994; Chacon et al., 2005). Paradoxically, caloric deficit increases basal and restraint stress-induced plasma corticosterone (CORT) levels in the same animals (Akana et al., 1994; Chacon et al., 2005), evidence that fasting "uncouples" anxiety-like behavior and ACTH release from CORT responses. It is posited that the benefits of this are three-fold: first, downregulated central CRH signaling attenuates its anorexigenic effects (Krahn et al., 1986; Krahn et al., 1988; Rothwell, 1990), limiting the ability of stress to suppress food intake during periods of negative energy balance. Second, increased CORT facilitates energy mobilization to support glucose-dependent processes, despite negative energy balance (Dallman et al., 1999). Third, attenuated anxiety may promote foraging in threatening environments, increasing the likelihood of encountering food. Although metabolic tuning of stress responses has clear adaptive value, the underlying neural mechanisms by which this shift occurs remain unclear.

We propose that the ability of caloric deficit to decrease stress hypophagia, central drive to the HPA axis, and anxiety-like behavior depends on reduced signaling from prolactin-releasing peptide (PrRP)-positive noradrenergic (NA) A2 neurons and glucagon-like peptide-1 (GLP-1)-positive neurons in the caudal nucleus of the solitary tract (cNTS). NA/PrRP and GLP-1 neurons receive substantial visceral sensory input (Rinaman, 2007), allowing them to readily monitor the interoceptive environment. In turn, these neurons project to brain regions integral for HPA axis activation, anxiety-like behavior, and suppression of food intake (Rinaman, 2010,

2011). Loss of signaling from GLP-1 or NA/PrRP neurons substantially attenuates stress-induced HPA axis activation, hypophagia, and anxiety-like behavior (Cecchi et al., 2002; Kinzig et al., 2003; Rinaman, 2003a; Ritter et al., 2003; Rinaman and Dzmura, 2007; Schiltz and Sawchenko, 2007; Bienkowski and Rinaman, 2008; Zheng and Rinaman, 2013), and we recently reported that overnight fasting prevents visceral stress-induced activation of hindbrain GLP-1 and A2 NA neurons (Maniscalco and Rinaman, 2013).

Considering this, we hypothesize that cognitive stressors activate hindbrain GLP-1 and NA/PrRP neurons in rats fed *ad libitum*. We propose that overnight fasting attenuates cognitive stress-induced activation of GLP-1 and NA/PrRP neurons in conjunction with reduced anxiety-like behavior and reduced neural activation within the mpPVN and bed nucleus of the stria terminalis (BST), forebrain regions critical for HPA axis activation and anxiogenesis. Third, we predict that basal and cognitive stress-induced plasma CORT concentrations will be elevated in fasted rats, despite reduced mpPVN activation. Finally, we propose that pharmacological blockade of central GLP-1 receptor signaling will attenuate the hypophagic effects of cognitive stress in rats fed *ad libitum*.

3.2 MATERIALS & METHODS

Animals

Adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were housed singly in hanging stainless steel wire mesh cages in a temperature-controlled room (20-22°C) on a 12/12 hr light/dark cycle (lights off at 1900 hr). Rats had *ad libitum* (ad lib) access to pelleted chow (Purina 5001) and water, except as noted. All 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.

Experiment 1: Fasting-induced changes in neural cFos responses to cognitive stressors

Rats (225-275g BW) were given ad lib chow access, or were deprived of food (but not water) for 16-18 hr overnight in their home cage (DEP) prior to stressor treatment. On the day of the experiment rats were removed from their home cage between 0830 and 1030 hr and were either restrained for 30 min in a perforated plexiglass tube (RES; n = 4 ad lib; n = 8 DEP), or were placed on a brightly lit (~550 lux) elevated platform (45 X 10 cm with 1-cm-high edging, 90 cm above floor) for 5 min (EP; n = 6 ad lib; n = 6 DEP). Additional nonhandled (NH) control rats (n = 6 ad lib; n = 7 DEP) remained undisturbed in their home cages during the same time period.

Ninety minutes after stressor onset, rats were deeply anesthetized with pentobarbital sodium (39 mg/1.0 ml i.p., Fatal Plus Solution; Butler Schein) and perfused transcardially with a brief saline rinse followed by fixative (100 ml of 2% paraformaldehyde and 1.5% acrolein in 0.1M phosphate buffer, followed by 100 ml of 2% paraformaldehyde alone). Brains were post-fixed *in situ* overnight at 4°C, then removed from the skull and cryoprotected for 24-48 hr in 20% sucrose. Brains were blocked and sectioned coronally (35μm) using a Leica freezing-stage sliding microtome. Tissue sections were collected in six serial sets, and stored at -20°C in cryopreservant solution (Watson et al., 1986) until immunohistochemical processing.

Immunohistochemistry

Primary and secondary antisera were diluted in 0.1M phosphate buffer containing 0.3% Triton X-100 and 1% normal donkey serum. Two sets of tissue sections from each rat were incubated in

a rabbit polyclonal antiserum against cFos (1:20,000; EMD Chemicals, PC38), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch). Sections were then treated with Elite Vectastain ABC reagents (Vector Laboratories) and reacted with diaminobenzidine (DAB) intensified with nickel sulfate to produce a blue-black nuclear cFos reaction product. To visualize cFos within hindbrain GLP-1 neurons, one set of cFos-labeled tissue sections was subsequently incubated in a rabbit polyclonal antiserum against GLP-1 (1:10,000; Bachem, T-4363), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch), Elite Vectastain ABC reagents (Vector), and reacted with plain DAB to produce a brown cytoplasmic reaction product.

The second set of cFos immunoperoxidase-labeled tissue sections was used to simultaneously visualize cFos expression by dopamine beta hydroxylase (DβH-) and PrRP-positive A2 neurons in the cNTS, and within the PrRP terminal-rich region of the anterior ventrolateral (vl)BST. For this purpose, sections were incubated in a cocktail of mouse anti-DβH (1:5,000; Millipore, MAB308) and rabbit anti-PrRP (1:1,000; Phoenix Pharmaceuticals, H-008-52), followed by a cocktail of AlexaFluor 488-conjugated donkey anti-mouse IgG (1:300, Jackson ImmunoResearch) and Cy3-conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch) to produce green and red fluorescent cytoplasmic signals, respectively.

A third set of tissue sections from each rat was used to visualize cFos within the GLP-1 terminal-dense mpPVN. Tissue sets were first processed for GLP-1 immunoperoxidase labeling, as described above. Reacted sections were then incubated in one of two rabbit antisera raised against cFos protein: one was provided by Dr. Philip Larsen, Denmark (1:5,000) and the second was purchased from EMD Chemicals (1:2,000; PC38). Statistical comparisons confirmed that these cFos antisera produced similar results. Tissue sections were then incubated in Cy3-

conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch) to produce a red fluorescent nuclear signal localizing cFos protein.

Imaging & Quantification of cFos expression by GLP-1 neurons

GLP-1 neurons were visualized using a light microscope and 20X/40X objectives to determine the number of GLP-1-positive neurons and the proportion that were double-labeled for cFos. GLP-1 neurons were counted bilaterally within the cNTS and adjacent reticular formation through the entire rostrocaudal extent of both GLP-1 cell groups (i.e., from the cervical spinal cord through the cNTS just rostral to the area postrema (AP); ~15.46 mm to 13.15 mm caudal to bregma). Criteria for counting a neuron included brown GLP-1 cytoplasmic labeling and a visible nucleus; double-labeled GLP1 neurons were those that displayed visible blue-black nuclear cFos immunolabeling, regardless of intensity. cFos-positive GLP-1 neurons were represented as a proportion of total GLP-1 neurons counted within the cNTS and/or reticular formation.

Imaging & Quantification of cFos expression by $PrRP^+$ and $PrRP^-$ A2 neurons

$PrRP^+$ and $PrRP^-$ A2 neurons (all of which were DβH-positive) were imaged using a 20X objective on an Olympus microscope equipped for brightfield and epifluorescence illumination, and photographed using a digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Neurons were counted in photographic images using Adobe Photoshop CS4 image software. Criteria for counting a neuron included clear cytoplasmic DβH labeling and a visible nucleus. $PrRP^+$ A2 neurons were also immunopositive for cytoplasmic PrRP, whereas $PrRP^-$ A2 neurons were not. Neurons were considered cFos positive if their nucleus contained blue/black cFos

immunoperoxidase labeling, regardless of intensity. Single-, double-, and triple-labeled neurons were counted bilaterally through the rostrocaudal extent of the A2 cell group (i.e., from the cervical spinal cord through the cNTS just rostral to the AP; ~15.46 mm to 13.15 mm caudal to bregma). In each analyzed case, cFos-positive A2 neurons that were $PrRP^+$ or $PrRP^-$ were represented as a percent of total $PrRP^+$ and $PrRP^-$ A2 neurons counted, respectively.

Imaging & Quantification of cFos expression in the mpPVN

Forebrain tissue sections labeled for GLP-1 immunoperoxidase and cFos immunofluorescence were viewed on the Olympus photomicroscope described above. Using a 10X objective, photographic images were captured from a single selected rostrocaudal level through the mpPVN (~ 1.78 mm caudal to bregma). This selected level was characterized by dense GLP-1 terminal labeling that clearly defined the boundaries of the mpPVN (e.g., see Figure 4). cFos-positive neurons within this defined region were counted bilaterally on captured images using Adobe Photoshop CS4 image software. The criterion for counting a neuron as cFos-positive was the presence of visible red fluorescent nuclear immunolabeling, regardless of intensity.

Imaging & Quantification of cFos expression in the PrRP terminal-dense region of the anterior vlBST

Forebrain tissue sections labeled for cFos immunoperoxidase and PrRP/DβH immunofluorescence were viewed on the Olympus photomicroscope described above. Using a 10X objective, photographic images were captured from a single selected rostrocaudal level of the anterior vlBST (~ 0.3 mm caudal to bregma) that is characterized by particularly dense PrRP and DβH terminal labeling (see Figure 9). Using Adobe Photoshop CS4 image software, a region

of interest (ROI) was mapped around the densest PrRP terminal labeling bilaterally by an investigator blinded to experimental group. cFos-positive neurons were then counted bilaterally within the defined ROI, which largely corresponded to the fusiform subnucleus of the anterior vlBST. The criterion for counting cFos-positive neurons within each ROI was the presence of blue-black nuclear immunoperoxidase labeling, regardless of intensity. Within the ROI, number of cFos-positive neurons per 100 μm^2 was quantified and averaged bilaterally in each rat.

Statistics

Paired samples t-tests were used to compare the proportion of GLP-1 neurons located within the reticular formation versus the cNTS, and to compare cFos activation percentages between reticular and cNTS GLP-1 neurons following each experimental treatment. Two-way multivariate ANOVA was used to reveal main effects of and interactions between stress treatment (NH vs. RES vs. EP) and feeding status (ad lib vs. DEP) on cFos activation of GLP-1 neurons, $PrRP^+$ A2 neurons, $PrRP^-$ A2 neurons, mpPVN neurons, and vlBST neurons. When F-values indicated significant main or interaction effects, ANOVAs were followed by Fisher's LSD post hoc analyses. Differences were considered significant when $p < .05$. Pearson's R correlation coefficient was used to determine whether significant correlations existed between the proportion of GLP-1 neurons activated within the reticular formation and the cNTS.

Experiment 2: Fasting-induced changes in anxiety-like behavior

Rats (225-275g BW) were deprived of food (but not water) for 16-18 hr overnight (n = 4) or were fed ad lib (n = 4) prior to behavioral assessment. On the day of the experiment rats were removed from their home cage between 0830 and 1030 hr and tested for anxiety-like behavior on

the elevated plus maze (EPMZ). The EPMZ is a validated test of behavioral anxiety in rats that measures approach-avoidance conflict generated by the motivation and fear associated with exploring novel environments (Montgomery, 1955; Pellow et al., 1985). The EMPZ consisted of two open arms (45 X 10 cm, ~ 550 lux) with transparent 1 cm-high edging, and two closed arms (45 X 10 X 48 cm, ~ 12 lux) extending from a common central platform (10 X 10 cm) elevated 90 cm above the floor. The EPMZ was located in a quiet, evenly lit behavioral testing room immediately adjacent to the animal housing room. Behavior on the EPMZ was videotaped for 5 min, and then rats were returned to their home cage. The EPMZ was cleaned with a mild odor-neutralizing cleanser and allowed to dry between rats. Tapes were later manually scored by an investigator blinded to experimental group to determine open arm time, closed arm time, center time, open arm entries, closed arm entries, and total entries, according to previously described procedures (Walf and Frye, 2007). A rat was considered to have entered an open or closed maze arm when all four paws initially occupied it. A significant decrease in open arm time and/or open arm entries or an increase in closed arm time and/or closed arm entries is interpreted as increased anxiety-like behavior (Pellow et al., 1985). Additionally, an automated software system (ANY-maze, Stoelting Co.) was used to determine total distance traveled by each rat during the 5 min EPMZ test.

Statistics

Independent-samples t-tests were used to reveal differences between ad lib and DEP rats on open arm time, closed arm time, center time, open arm entries, closed arm entries, total entries, and total distance traveled. Differences were considered significant when $p < .05$.

Experiment 3: Fasting-induced changes in CORT response to cognitive stress

CORT responses to restraint (RES)

Rats (225-275g BW) were deprived of food (but not water) for 16-18 hr overnight (n = 5) or were fed ad lib (n = 6) prior to RES. On the day of the experiment rats were removed from their home cage between 0830 and 1030 hr and were immediately placed into perforated Plexiglass RES tubes. Using a within-subjects design, blood was sampled from the tail vein within 90 seconds of placement in the restrainer (baseline sample) and again after 30 minutes of RES (peak sample), after which rats were removed from the restrainers and returned to their home cage. Thirty minutes following return to the home cage (i.e., 60 minutes following onset of RES stress), rats were decapitated and trunk blood was collected (recovery sample).

CORT responses to elevated platform (EP) exposure

Rats were deprived of food (but not water) for 16-18 hr overnight (n = 14) or were fed ad lib (n = 16) prior to EP exposure. On the day of the experiment rats were removed from their home cage between 0830 and 1030 hr and were immediately placed on a brightly lit elevated platform for 5 minutes, as described above. Using a between-subjects design, rats were decapitated and trunk blood was collected either 15 min (peak sample, n = 6 ad lib; n = 5 DEP) or 35 minutes (recovery sample, n = 4 ad lib; n = 4 DEP) after the onset of the 5 min EP exposure. Peak sample timing was selected based on a prior report that platform exposure maximally increases plasma CORT concentrations within 15 min (Kinzig et al., 2003), and the recovery sample timepoint was chosen to match the duration of home cage recovery in the cohort of rats that received RES stress.

Corticosterone Enzyme Immunoassay

All blood samples were collected into EDTA-coated tubes and placed immediately on ice. Blood samples were then centrifuged (5500 rpm, 10 min, 4°C), and plasma was removed and stored at -80°C until processing. Plasma CORT concentrations were determined using a competitive enzyme immunoassay (Immunodiagnostic Systems Ltd., Fountain Hills, AZ), according to the manufacturer's instructions. Assay sensitivity for CORT was 0.55ng/ml, with synthetic CORT recovery of 98.6 ± 2.8% and linearity of 99.7 ± 1.78%.

Statistics

Two-way ANOVA with repeated measures was used to reveal main effects of and interactions between sample time (baseline vs. peak vs. recovery) and feeding status (ad lib vs. DEP) on plasma CORT concentration during and after RES stress (within-subjects design). When F-values indicated a significant effect, the ANOVA was followed by post hoc tests with Bonferroni correction for multiple comparisons. Differences were considered significant when $p \leq .05$.

Two-way ANOVA was used to reveal main effects of and interactions between sample time (baseline vs. peak vs. recovery) and feeding status (ad lib vs. DEP) on plasma CORT concentration following EP exposure. Baseline samples used for the between-subjects EP comparisons were those collected from the cohort of rats sampled at the outset of RES. When F-values indicated significant main or interaction effects, ANOVAs were followed by Fisher's LSD post hoc analyses. Differences were considered significant when $p \leq .05$.

Experiment 4: Role of central GLP-1 signaling in stress-induced hypophagia

Cannulation Procedures

Rats (290-310 g BW) were anesthetized by inhalation of isoflurane (Halocarbon Laboratories, River Edge, NJ, USA; 1-3% in oxygen) and placed into a stereotaxic frame in the flat-skull position. Rats were fitted with chronic indwelling 26-gauge stainless steel guide cannulas (Plastics One) aimed at the lateral ventricle. Guide cannulae were positioned 1.4 mm lateral and 0.9 mm caudal to bregma, with the cannula tip protruding 2.7 mm below the surface of the skull. Cannulae were fixed to the skull with anchor screws and dental acrylic and fitted with removable obturators that extended to the tip of the cannula. Rats were allowed to recover for 4-5 days following surgery, at which point all rats exceeded their pre-surgery BW.

Hypophagia Experiments

Beginning 4-5 days after surgery, rats (n = 16) were acclimated for 3 days to mock cannula injections at 1800 hr. This included gentle restraint and obturator manipulation, intended to mimic the injection procedure except for infusions. On each food intake measurement day, rats were deprived of food (but not water) for 2 hr prior to dark onset. The following treatments were administered in a counterbalanced design: 1) i.c.v. saline (3 µl over 1.5 min), 2) i.c.v. saline + RES, 3) i.c.v exendin-(9-39) (Ex9; a specific GLP-1 receptor antagonist; Tocris Bioscience, 2081; 100 µg in 3 µl saline over 1.5 min), and 4) i.c.v. Ex9 + RES. I.c.v. saline or Ex9 was administered 15 min prior to the 30 min RES, which was completed just before lights out. At dark onset (1900 hr), pre-weighed pelleted chow was placed on the home cage floor, and intake was measured to the nearest 0.1 g at 30 and 60 min (corrected for spillage beneath the cage). Baseline intake was measured in the same manner the day before each experimental treatment

day. The amount of food consumed was represented as percent of BW to correct for small between-animal BW differences. Each rat received 2 of the 4 possible treatment combinations, but no rat received the same i.c.v. injection or RES stress more than once. Thus, for statistical purposes, data obtained for each treatment group condition were considered independent and were analyzed according to a between-subjects rather than within-subjects design.

Evaluation of Cannula Placement

Correct cannula placement was verified within 1 week after feeding data collection. For this purpose, water-replete rats were injected i.c.v. with 2 μl of sterile saline containing 5 ng of angiotensin II (AngII; Bachem, H-1705). Only data from rats that drank at least 5ml of water within 30 min after AngII injection were included in the study.

Statistics

Two-way ANOVA with repeated measures (time) was used to reveal main effects of and interactions between experimental treatment group (i.e., baseline, i.c.v. saline, i.c.v. saline + RES, i.c.v. Ex9, and i.c.v. Ex9 + RES) and time (0-30 min, 30-60 min, and 0-60 min) on chow intake. When F-values indicated significant main effects or interactions, the ANOVA was followed by post hoc tests with Bonferroni correction for multiple comparisons. Differences were considered significant when $p < .05$.

3.3 RESULTS

Experiment 1: Fasting-induced changes in neural cFos responses to cognitive stressors

Feeding status and/or stress treatment did not affect the total number of immunoreactive hindbrain GLP-1, $PrRP^+$, or $PrRP^-$ A2 neurons. Two-way ANOVA did, however, reveal significant main effects of and interactions between stressor treatment and feeding status on cFos expression by hindbrain GLP-1 neurons, $PrRP^+$ A2 neurons, $PrRP^-$ A2 neurons, and within the vlBST (Table 3). There also was a significant main effect of stress treatment on cFos expression within the mpPVN, but no main effect of feeding status and no interaction effect.

Table 3. Multivariate ANOVA Statistics

Multivariate ANOVA main effects, interactions, and *p*-values for cFos counts in ad lib fed or DEP rats following NH, Restraint, and Elevated Platform.

Effect Source	Dependent Variable	F	*p* value
Treatment [*F*(2,31)]	Activation of GLP-1 Neurons (%)	43.52	$p < .001$*
NH vs. Restraint vs. Elevated Platform	Activation of PrRP$^+$ A2 Neurons (%)	36.79	$p < .001$*
	Activation of PrRP$^-$ A2 Neurons (%)	45.06	$p < .001$*
	cFos$^+$ Neurons in mpPVN	21.09	$p < .001$*
	cFos$^+$ Neurons per 100 µm^2 in vlBST	71.25	$p < .001$*
	Number of GLP-1 Neurons per Section	0.05	$p > .05$
	Number of PrRP Neurons per Section	2.96	$p > .05$
Feeding Status [*F*(1,31)]	Activation of GLP-1 Neurons (%)	800.97	$p < .001$*
Ad lib vs. DEP	Activation of PrRP$^+$ A2 Neurons (%)	175.87	$p < .001$*
	Activation of PrRP$^-$ A2 Neurons (%)	29.80	$p < .001$*
	cFos$^+$ Neurons in mpPVN	3.51	$p = .07$
	cFos$^+$ Neurons per 100 µm^2 in vlBST	16.89	$p < .001$*
	Number of GLP-1 Neurons per Section	0.02	$p > .05$
	Number of PrRP Neurons per Section	0.89	$p > .05$
Interaction [*F*(2,31)]	Activation of GLP-1 Neurons (%)	41.79	$p < .001$*
Treatment X Feeding Status	Activation of PrRP$^+$ A2 Neurons (%)	10.88	$p < .001$*
	Activation of PrRP$^-$ A2 Neurons (%)	6.45	$p < .01$*
	cFos$^+$ Neurons in mpPVN	2.50	$p > .05$
	cFos$^+$ Neurons per 100 µm^2 in vlBST	3.85	$p < .05$*
	Number of GLP-1 Neurons per Section	0.28	$p > .05$
	Number of PrRP Neurons per Section	0.26	$p > .05$

*statistically significant

GLP-1 neuronal distribution and activation

Similar to our recent report examining the effect of fasting on visceral stress-induced neural activation (Chapter 2), a moderate proportion (~ 32%) of GLP-1 neurons were $cFos^{+}$ in ad lib-fed, NH control rats. Conversely, GLP-1 neurons did not express cFos in NH rats following DEP (Figure 6E). Compared to baseline activation, RES and EP stress each significantly increased the proportion of GLP-1 neurons expressing cFos in ad lib-fed rats, with a greater degree of activation induced by EP compared to RES (Figure 6E). However, GLP-1 neurons were not activated in DEP rats after either RES or EP stress.

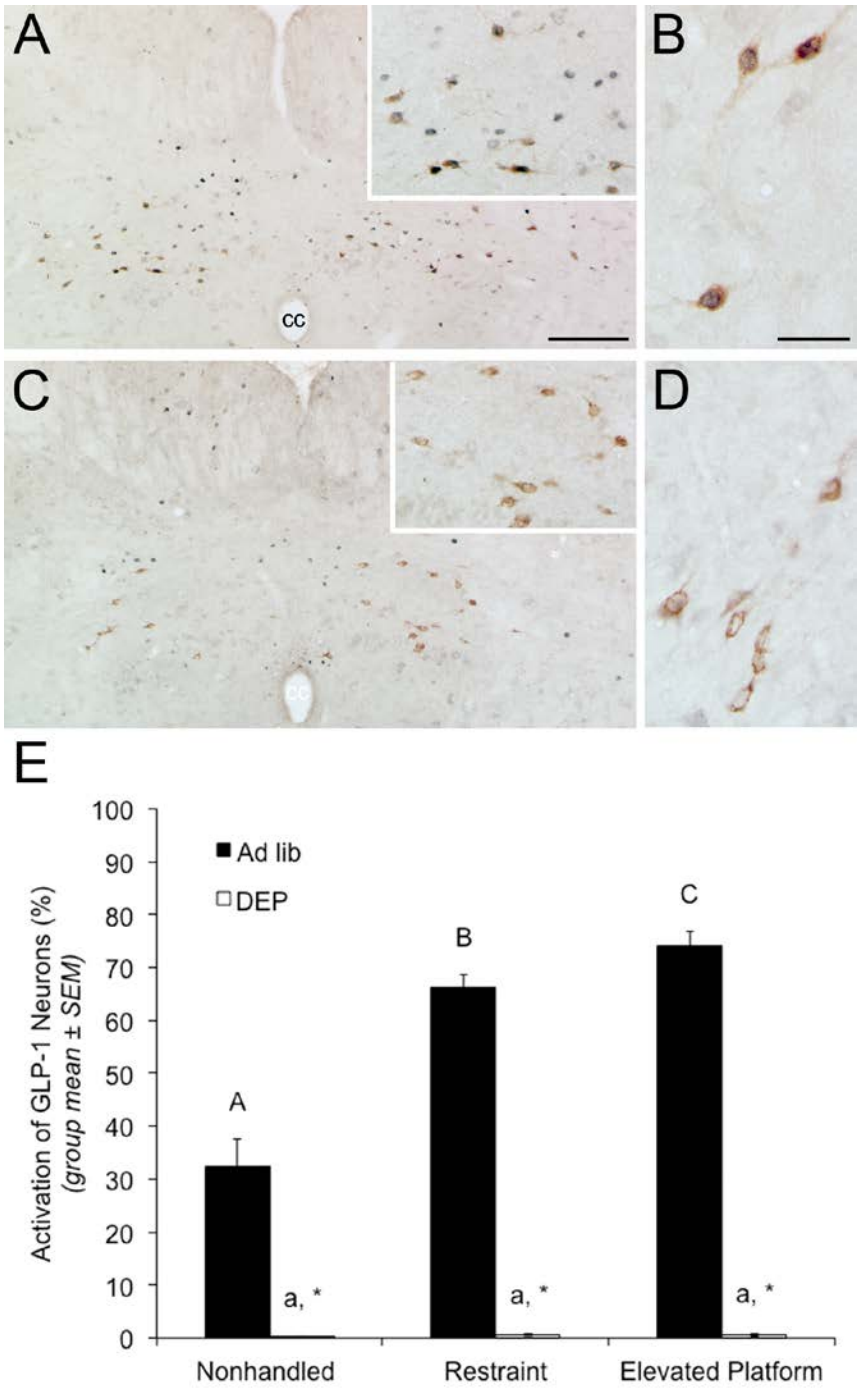

Figure 6. Overnight fasting eliminates cognitive stress-induced cFos activation of GLP-1 neurons

Representative color images depicting neuronal cFos expression (black nuclear label) within GLP-1^{+} neurons (brown cytoplasmic label) in the cNTS (A, C) and reticular formation (B, D) in rats exposed to elevated platform stress. A, robust cFos activation within the cNTS (~14.36 mm caudal to bregma) in an ad lib-fed rat. *Inset*, higher

magnification view of several cFos+ GLP-1 neurons. B, $cFos^{+}/GLP\text{-}1^{+}$ neurons within the reticular formation in an ad lib-fed rat. C, cNTS $GLP\text{-}1^{+}$ neurons do not express cFos in a fasted rat after elevated platform exposure. *Inset*, higher magnification view of several $GLP\text{-}1^{+}$ neurons, none of which express cFos. D, reticular GLP-1 neurons do not express cFos in a fasted rat after elevated platform exposure. E, summary data illustrating the proportion of double-labeled neurons (i.e., both $cFos^{+}$ and $GLP\text{-}1^{+}$) within the cNTS and adjacent reticular formation in ad lib-fed rats (solid bars) or fasted rats (DEP; open bars) after no manipulation (nonhandled), restraint stress, or elevated platform stress. See Table 3 for two-way ANOVA results. Fasting completely eliminated cFos expression by GLP-1 neurons. Asterisks indicate significantly reduced ($p < 0.05$) GLP-1 cFos expression in DEP vs. ad lib-fed rats in the same treatment group. Within the same feeding status group (i.e., ad lib or DEP), bars with different letters are significantly different ($p < 0.05$). cc, central canal. Scale bar in A = 200 μm, applies also to C. Scale bar in B = 50 μm, applies also to D.

A t-test for paired samples demonstrated that the proportion of all GLP-1 neurons located within the cNTS (56.55 ± 1.37%) was significantly greater than the proportion of located within the reticular formation (43.45 ± 1.37%), [t (36) = 4.775, $p < .001$]. However, there were almost no significant differences between the proportion of cNTS and reticular GLP-1 neurons that expressed cFos after each combination of experimental treatments (Table 4). The one exception was a small but statistically significant difference in the distribution of activated neurons after RES in DEP rats, in which activation was very low overall but was significantly higher among the reticular population. Activation of cNTS GLP-1 neurons correlated strongly with activation of reticular GLP-1 neurons across all stress treatment conditions and both feeding groups (R = 0.972, $p < .001$), and also selectively within ad lib-fed rats (R = 0.917, $p < .001$). Activation of GLP-1 neurons among DEP rats was so low that the absence of a correlation within this feeding group was not surprising.

Table 4. Paired-samples t-test statistics.

Paired-samples t-tests comparing mean differences between percent cFos activation of NTS GLP-1 neurons and reticular GLP-1 neurons following each experimental treatment combination.

		NTS		Reticular				
Feeding Status	**Treatment**	**Mean**	**SEM**	**Mean**	**SEM**	**t (df)**	***p* value**	**n**
Ad lib	Nonhandled	33.80	6.69	30.55	4.01	0.787 (5)	$p > .05$	6
	Restraint	69.91	1.95	61.08	4.39	2.503 (3)	$p > .05$	4
	Elevated Platform	73.90	2.97	74.82	3.37	0.289 (5)	$p > .05$	6
DEP	Nonhandled	0.26	0.26	0.00	0.00	1.000 (6)	$p > .05$	7
	Restraint	0.00	0.00	1.52	0.48	3.133 (7)	$p < .05$*	8
	Elevated Platform	0.14	0.14	1.02	0.80	1.032 (5)	$p > .05$	6

*statistically significant

A2 neuronal activation

Similar to GLP-1 neurons, ad lib-fed rats displayed moderate cFos activation of $PrRP^+$ A2 neurons under NH control conditions (~28%), and significantly increased activation after either RES or EP (Figure 7C). Markedly smaller proportions of $PrRP^-$ A2 neurons expressed cFos under NH control or stress conditions (Figure 7D). In contrast to the increased effectiveness of EP vs. RES in activating GLP-1 neurons in ad lib-fed rats (Figure 7E), RES activated significantly larger proportions of $PrRP^+$ and $PrRP^-$ A2 neurons than were activated by EP in ad lib-fed rats (Figure 7C, D). Similar to the effect of DEP to virtually abolish GLP-1 neural activation under all treatment conditions (Figure 7E), DEP eliminated cFos expression by $PrRP^+$ and $PrRP^-$ A2 neurons under NH control conditions and after EP stress (Figure 7C, D). Compared to activation in ad lib-fed rats, DEP significantly attenuated (but did not eliminate) the ability of RES to activate both $PrRP^+$ and $PrRP^-$ A2 neurons compared to activation under NH control conditions or after EP.

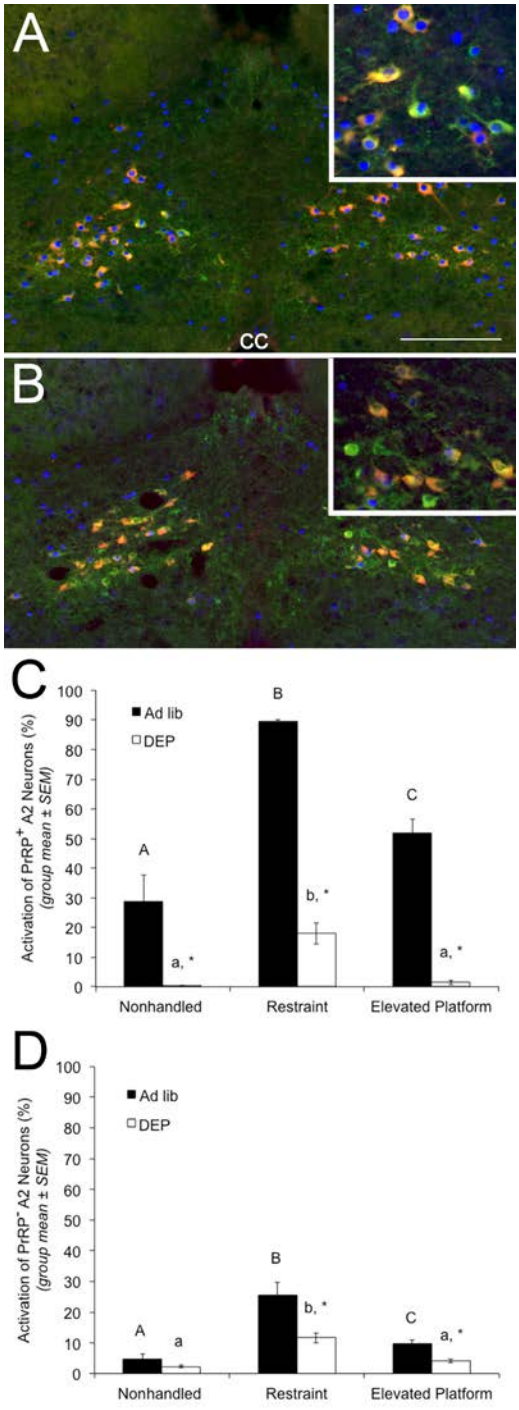

Figure 7. Overnight fasting reduces cFos activation within PrRP$^+$ and PrRP$^-$ A2 neurons

Representative color images depicting neuronal cFos expression (blue nuclear label) within PrRP$^+$ (red) and DβH$^+$ (green) neurons in the cNTS in rats exposed to restraint stress. A, robust cFos activation within the cNTS (~14.36

mm caudal to bregma) in an ad lib-fed rat, including activation of most $PrRP^{+}/D\beta H^{+}$ neurons and a smaller proportion of $PrRP^{-}/D\beta H^{+}$ neurons. *Inset*, higher magnification view of several $cFos^{+}/PrRP^{+}/D\beta H^{+}$ neurons. B, few $PrRP^{+}/D\beta H^{+}$ or $PrRP^{-}/D\beta H^{+}$ neurons express cFos in a fasted rat after restraint stress. *Inset*, higher magnification view of several $PrRP^{+}/D\beta H^{+}$ and $PrRP^{-}/D\beta H^{+}$ neurons, a few of which express cFos. C & D, summary data illustrating the proportion of triple-labeled (i.e., $cFos^{+}$, $PrRP^{+}$, $D\beta H^{+}$) or double-labeled (i.e., $cFos^{+}$, $PrRP^{-}$, $D\beta H^{+}$) cNTS neurons in ad lib-fed rats (solid bars) or fasted rats (DEP; open bars) after no manipulation (nonhandled), restraint stress, or elevated platform stress. C, Fasting eliminated cFos expression by $PrRP^{+}/D\beta H^{+}$ neurons in rats exposed to nonhandled control conditions or elevated platform stress, and significantly reduced cFos expression by $PrRP^{+}/D\beta H^{+}$ neurons in rats exposed to restraint. D, few $PrRP^{-}/D\beta H^{+}$ neurons expressed cFos in the nonhandled control condition, regardless of feeding status. Fasting attenuated stress-induced cFos expression in $PrRP^{-}/D\beta H^{+}$ neurons. See Table 3 for two-way ANOVA results. In C & D, asterisks indicate significantly reduced ($p < 0.05$) neural cFos expression in DEP rats vs. ad lib-fed rats in the same treatment group. Within the same feeding status group (i.e., ad lib or DEP), bars with different letters are significantly different ($p < 0.05$). cc, central canal. Scale bar in A = 200 μm, applies also to B.

mpPVN neuronal activation

Irrespective of feeding status, cFos activation within the mpPVN was increased to a similar degree following RES and EP stress as compared to NH controls (Table 3, Figure 8). A main effect of feeding status on cFos counts approached, but did not reach, statistical significance (p = .07; Table 3), and there was no significant interaction between feeding status and stress treatment on mpPVN cFos counts. Although this precluded post hoc comparisons between ad lib-fed and DEP rats in each stress treatment group, the data shown in Figure 8 are consistent with the hypothesis that DEP reduced cFos activation in the mpPVN under NH control conditions and after EP stress, but not after RES.

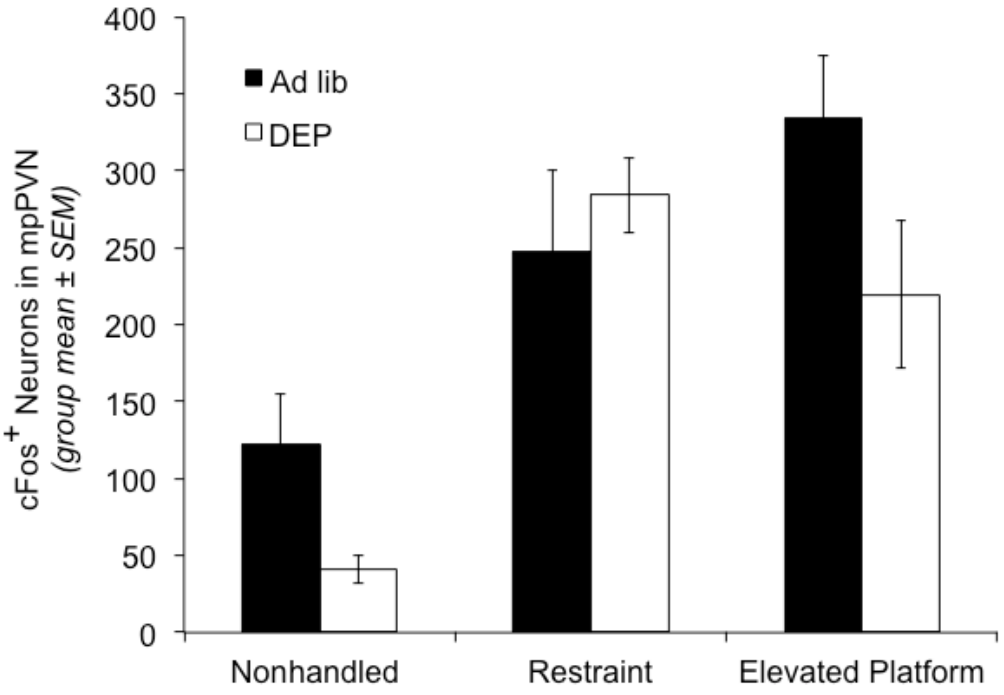

Figure 8. Overnight fasting does not attenuate cognitive stress-induced mpPVN cFos activation

Summary data illustrating the number of $cFos^+$ mpPVN neurons in ad lib-fed rats (solid bars) or fasted rats (DEP; open bars) after no manipulation (nonhandled), restraint stress, or elevated platform stress. See Table 3 for two-way ANOVA results. Compared to nonhandled control rats, either restraint or elevated platform exposure increased cFos expression within the mpPVN, regardless of feeding status. Despite the apparent trend towards less mpPVN cFos activation in fasted rats under nonhandled control conditions or after elevated platform exposure, there was no significant main effect of feeding status and no interaction between feeding status and stress treatment on mpPVN cFos activation.

Anterior vlBST neuronal activation

Low baseline levels of cFos expression were observed within the vlBST in NH control rats, regardless of feeding status (Figure 9C). RES and EP stress each significantly increased cFos activation in the vlBST, with RES stress evoking a larger increase than EP stress (Table 3, Figure 9C). The ability of RES or EP to activate cFos in the vlBST was modestly but significantly attenuated in DEP rats, in which RES-induced cFos activation remained significantly higher than activation after EP stress (Figure 9C).

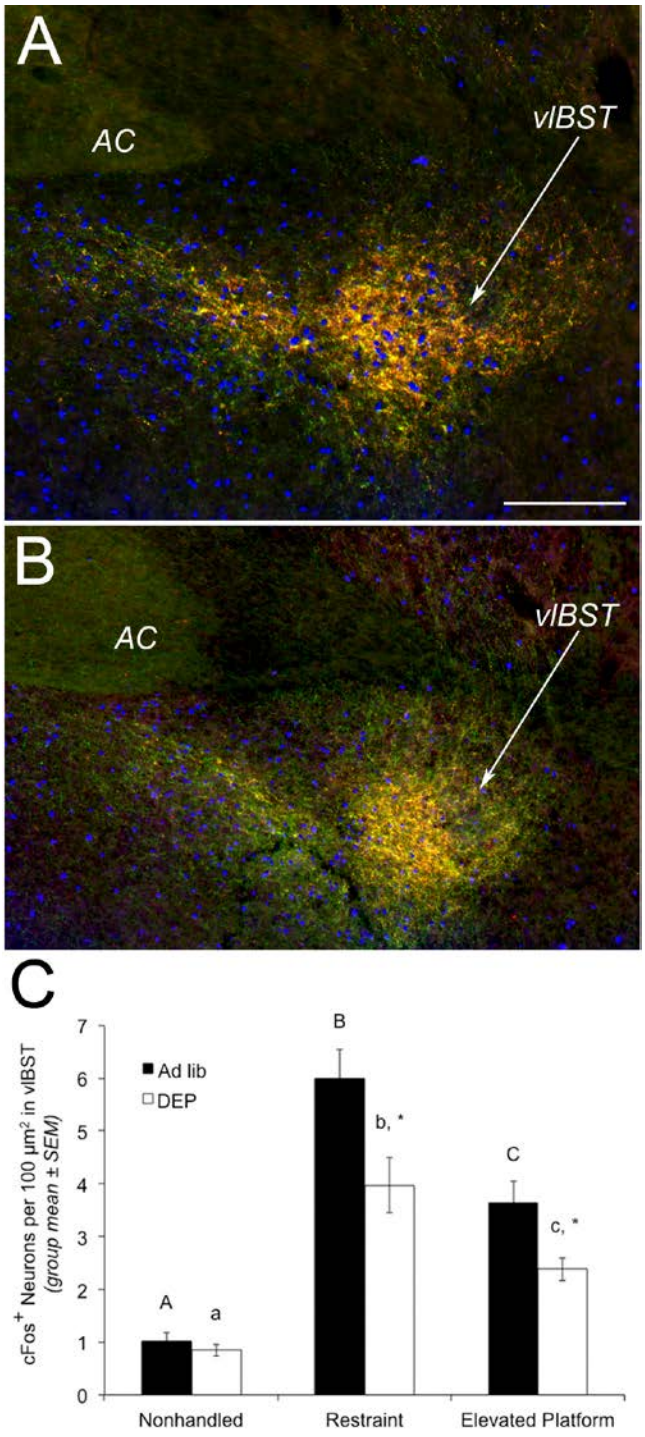

Figure 9. Overnight fasting reduces cFos activation within the anterior vlBST after cognitive stress

Fasting reduces cFos activation within the anterior vlBST after cognitive stress. Representative color images depicting neuronal cFos expression (blue nuclear label) within the PrRP (red) and DβH (green) terminal-rich region

of the vlBST (~0.26 mm caudal to bregma). A, in an ad lib-fed rat, many vlBST neurons express cFos after restraint. B, fewer vlBST neurons express cFos in a fasted rat after restraint. C, summary data illustrating the number of $cFos^+$ neurons per 100 μm^2 area of the PrRP/DβH terminal-rich vlBST in ad lib-fed rats (solid bars) or fasted rats (DEP; open bars) after no manipulation (nonhandled), restraint stress, or elevated platform stress. See Table 3 for two-way ANOVA results. Fasting did not affect baseline cFos but significantly attenuated stress-induced cFos expression in the vlBST. Asterisks indicate significantly reduced ($p < 0.05$) vlBST neural cFos expression in DEP rats vs. ad lib-fed rats in the same treatment group. Within the same feeding status group (i.e., ad lib or DEP), bars with different letters are significantly different ($p < 0.05$). AC, anterior commissure; vlBST, ventrolateral bed nucleus of the stria terminalis. Scale bar in A = 200 μm, applies also to B.

Experiment 2: Fasting-induced changes in anxiety-like behavior

Overnight DEP significantly attenuated anxiety-like behavior on the EPMZ (Figure 10). Independent-samples t-tests indicated that, as compared to ad lib-fed rats, DEP rats spent significantly more time in the EPMZ open arms [$t(6) = 5.599$, $p = .001$] and significantly less time in the EPMZ closed arms [$t(6) = 5.507$, $p = .002$]. There was no effect of feeding status on the amount of time spent in the EPMZ center zone [$t(6) = 1.223$, $p > .05$] (Figure 10A). DEP rats also made significantly more open arm entries [$t(6) = 9.000$, $p < .001$] and significantly fewer closed arm entries [$t(6) = 2.615$, $p < .05$] than ad lib-fed rats, with no between-group difference in the total number of arm entries [$t(6) = 0.747$, $p > .05$] (Figure 10B). There also was no effect of feeding status on total distance traveled during the 5-min EPMZ test (DEP rats = 16.98 ± 3.09 meters; ad lib-fed rats = 15.68 ± 0.26 meters), [$t(6) = 0.419$, $p > .05$].

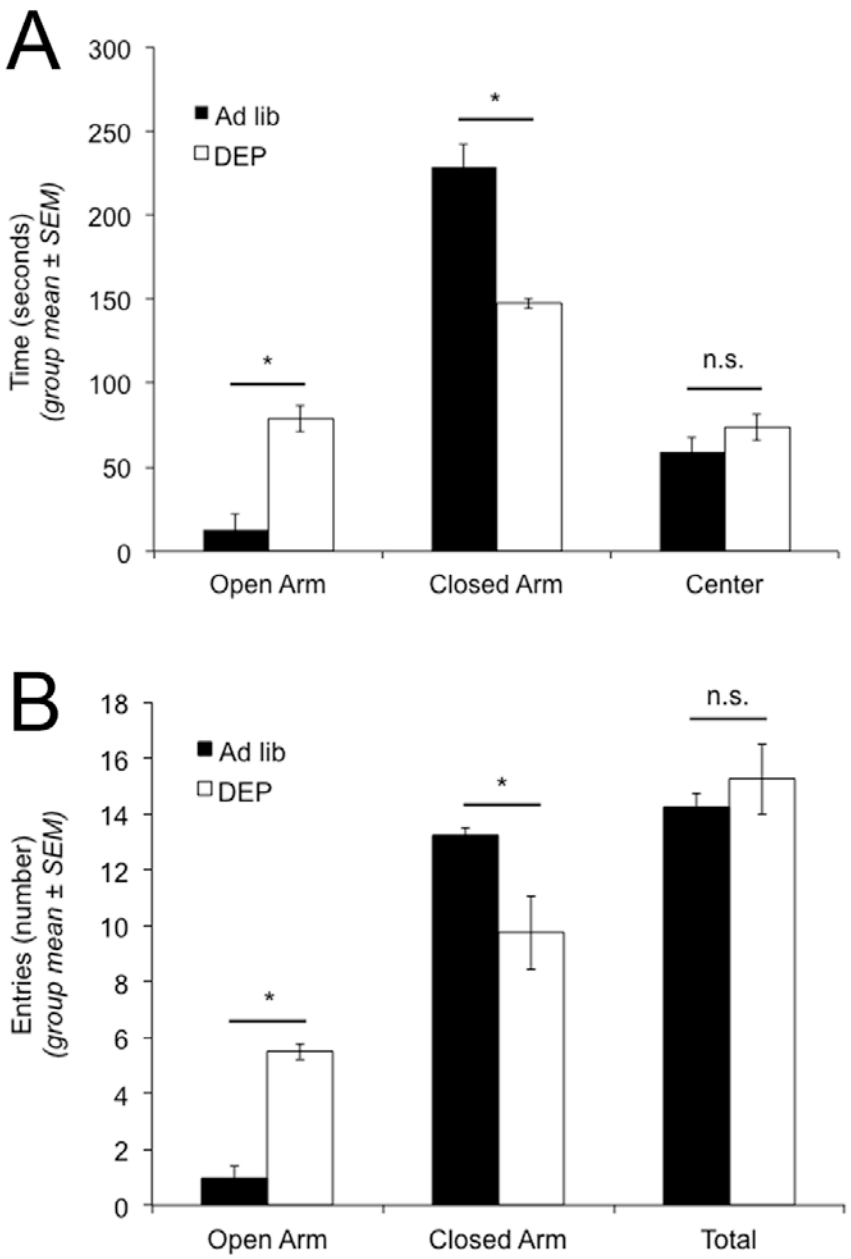

Figure 10. Overnight fasting attenuates anxiety-like behavior

Fasting attenuates anxiety-like behavior. A, summary data illustrating time spent in the open arms, closed arms, and center of the elevated plus maze by ad lib-fed rats (solid bars) or fasted rats (DEP; open bars). B, summary data illustrating the number of entries into the open arms, closed arms, or total arm entries on the elevated plus maze by ad lib-fed or fasted rats. Asterisks indicate significant differences between ad lib-fed vs. fasted groups ($p < 0.05$).

Experiment 3: Fasting-induced changes in CORT response to cognitive stress

Restraint (RES) stress

Two-way ANOVA with repeated measures revealed a significant main effect of feeding status on plasma CORT, [$F(1,9) = 11.478$, $p < 0.01$], such that overnight DEP significantly increased plasma CORT concentrations at baseline and in response to RES (Figure 11A). There also was a significant main effect of time on CORT concentrations [$F(2,18) = 59.5$, $p < 0.001$]. Post hoc analyses indicated that plasma CORT levels were lowest at baseline, were increased significantly at 30 min after RES onset (peak), and were significantly decreased 30 min after return to the home cage (recovery) (Figure 11A). The interaction between feeding status and time also was significant [$F(2,18) = 7.54$, $p < 0.01$]. In ad lib-fed rats, plasma CORT was significantly elevated at peak and then fell significantly during the recovery period. Conversely, in DEP rats, increased plasma CORT levels in the peak and recovery samples were statistically similar (Figure 11A). Pairwise comparisons indicated that plasma CORT levels in DEP rats were significantly higher than in ad lib-fed rats at baseline (ad lib rats = 5.89 ± 1.24 ng/ml; DEP rats = 39.9 ± 12.13 ng/ml), peak, and recovery (Figure 11A).

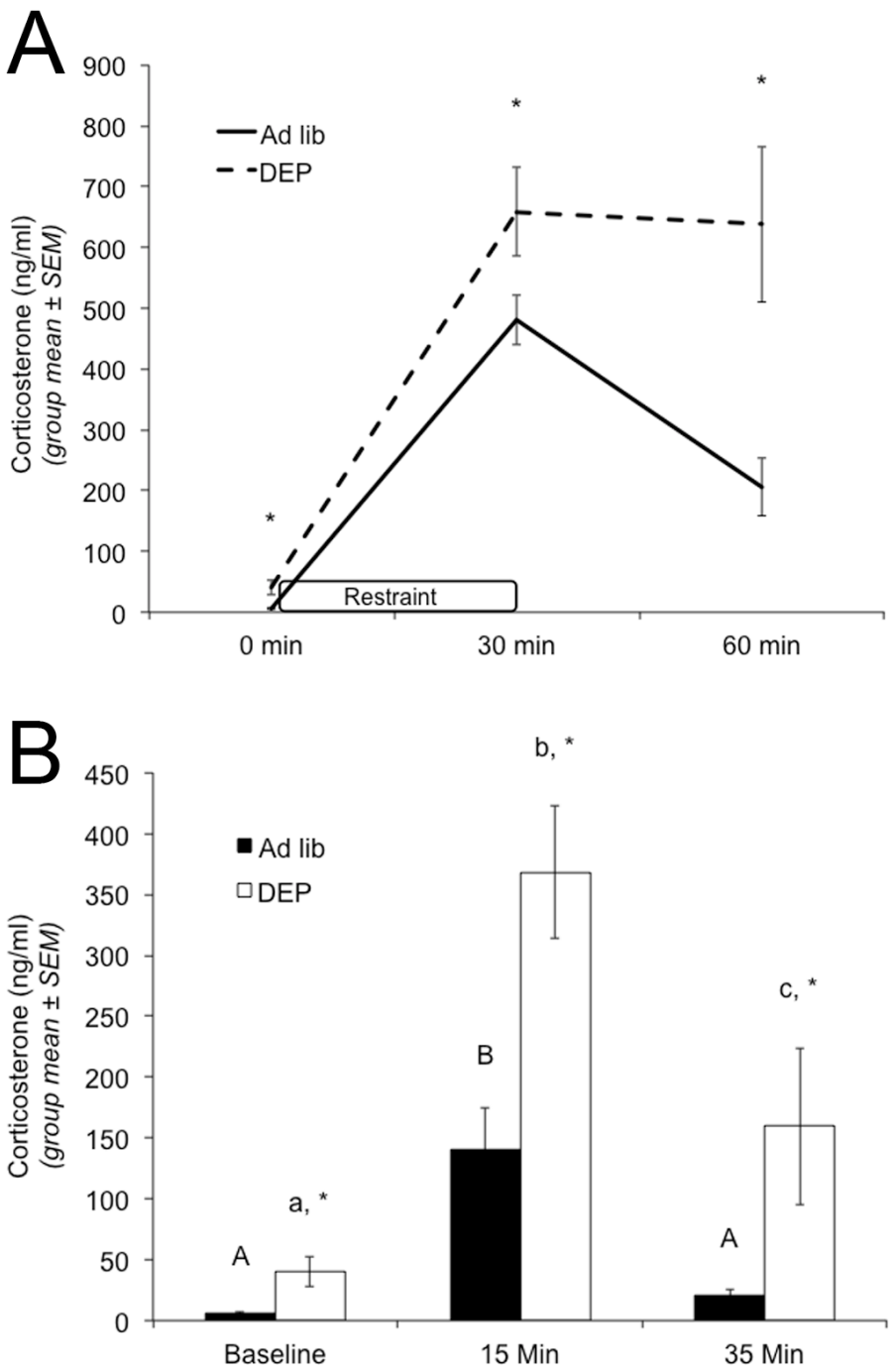

Figure 11. Overnight fasting increases plasma corticosterone levels in response to cognitive stress

Fasting increases plasma corticosterone levels at baseline and in response to cognitive stress. A, corticosterone levels in ad lib-fed (solid line) or fasted rats (DEP; dashed line) prior to restraint stress (0 min), at the end of restraint

stress (30 min), and 30 minutes after return to the home cage (60 min). Ad lib-fed rats displayed a significant increase in plasma CORT after 30 min of restraint stress, which decreased significantly by the 60 min recovery time point. Compared to ad lib-fed rats, fasted rats displayed significantly higher baseline (0 min) corticosterone levels, and a significantly larger increase after 30 min of restraint stress. Corticosterone levels remained significantly more elevated at the 60 min recovery time point in fasted vs. ad lib-fed rats. Asterisks at each time point indicate significant differences ($p < 0.05$) between fed and fasted rats. B, plasma corticosterone levels in ad lib-fed rats (solid bars) or fasted rats (DEP; open bars) at baseline (nonhandled), 15 min after the start of a 5-min elevated platform exposure (15 min), and 30 min after return to the home cage following elevated platform exposure (35 min). Ad lib-fed rats sacrificed at the 15 min time point displayed significantly higher plasma corticosterone levels compared to baseline, whereas ad lib fed rats sacrificed at the 35 min time point were similar to baseline levels. Plasma corticosterone levels were significantly elevated in fasted rats sacrificed at the 15 min time point, and remained significantly elevated in fasted rats sacrificed at the 35 min time point. Plasma corticosterone levels were significantly higher in fasted vs. fed rats at baseline (nonhandled), 15 min, and 35 min (astericks, $p < 0.05$). Within the same feeding status group (i.e., ad lib or DEP), bars with different letters are significantly different ($p < 0.05$).

Elevated platform (EP) stress

Two-way ANOVA indicated a significant main effect of feeding status on plasma CORT [$F(1,24) = 21.409$, $p < 0.001$], such that DEP rats had significantly higher overall plasma CORT levels than ad lib-fed rats (Figure 11B). ANOVA also revealed a main effect of sampling time on CORT levels [$F(2,24) = 25.216$, $p < 0.001$]. Post hoc analyses indicated that plasma CORT concentrations were significantly elevated 15 min after the onset of EP (peak) compared to CORT levels sampled at baseline or 30 min after return to the home cage (recovery). A feeding status X sampling time interaction was also significant [$F(2,24) = 4.203$, $p < 0.05$]. Overnight DEP significantly increased plasma CORT levels at baseline and in response to EP as compared to CORT responses in ad lib-fed rats (Figure 11B). In ad lib-fed rats, plasma CORT levels were significantly elevated in the peak sample group as compared to both the baseline and recovery sample groups, while CORT levels in the baseline and recovery sample groups did not differ. Conversely, in DEP rats, CORT levels in the recovery sample group remained significantly elevated compared to levels in the DEP baseline sample group.

Experiment 4: Role of central GLP-1 signaling in stress-induced hypophagia

Two-way ANOVA indicated significant main effects of treatment [$F(4,40) = 6.593$, $p < 0.001$] and time [$F(2,80) = 216.254$, $p < 0.001$] on food intake, along with a significant interaction effect [$F(8,80) = 11.329$, $p < .001$]. RES stress significantly reduced food intake during the first 30 min when rats were pre-treated with i.c.v. saline (Figure 12). Intake during the next 30 min period (i.e., 30-60 min) was not significantly reduced by restraint stress, although rats did not compensate for the initial period of hypophagia. Thus, total intake from 0-60 min remained suppressed after restraint stress in i.c.v. saline-treated rats compared to intake after all other

treatments. In the absence of restraint stress, i.c.v. administration of Ex9, the specific GLP-1 receptor antagonist, slightly but significantly increased intake during the first 30 min compared to baseline intake, but not compared to intake after i.c.v. saline alone (Figure 12). Subsequently, i.c.v. Ex9-treated rats slightly decreased intake during the second 30 min, as compared to baseline, such that 0-60 min intake in i.c.v. Ex9-treated rats was no different than baseline or i.c.v. saline. Ex9 pre-treatment significantly attenuated restraint-induced hypophagia during the first 30 min, and also from 0-60 min. Food intake was statistically similar in rats under baseline, i.c.v. saline, and i.c.v. Ex9 + RES conditions at all timepoints (Figure 12).

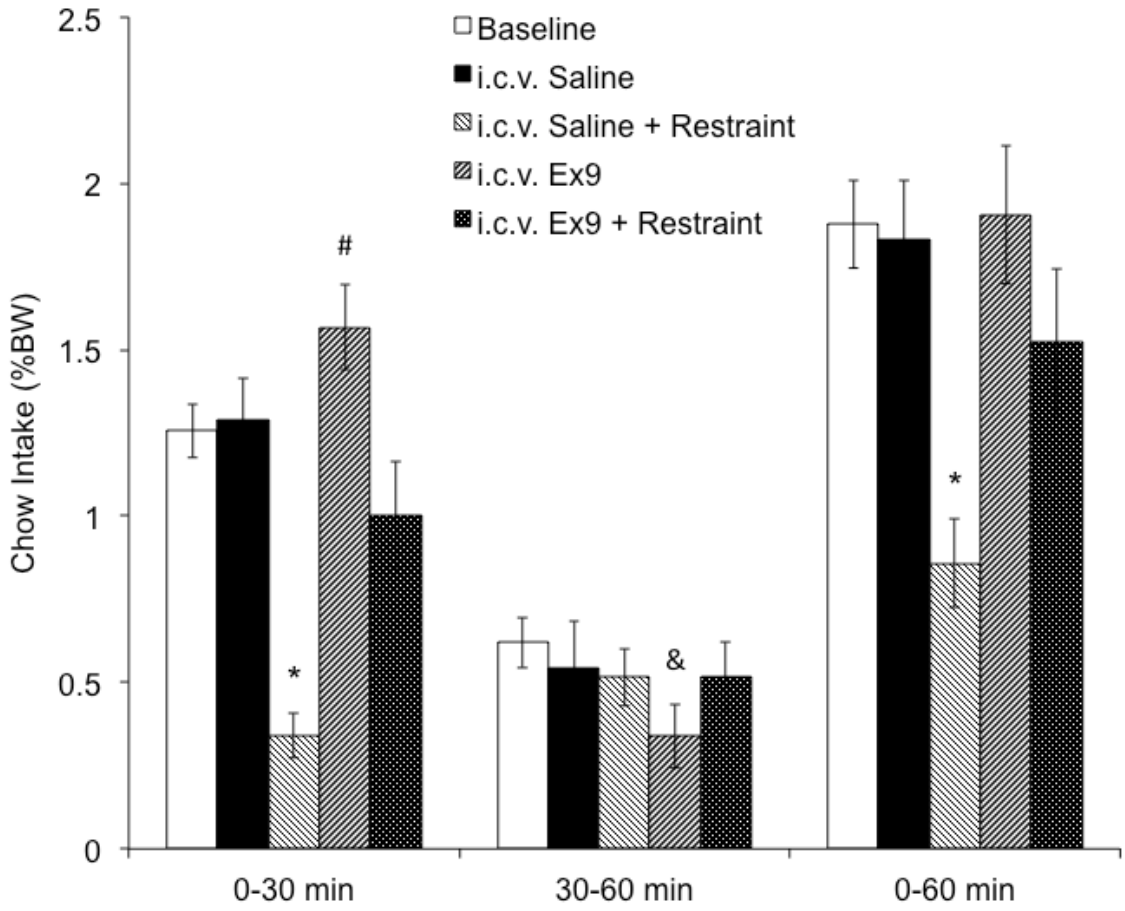

Figure 12. Central (lateral i.c.v.) GLP-1 receptor antagonism ameliorates restraint stress-induced hypophagia
Dark-onset chow intake (expressed as percentage of body weight) is illustrated during the first 0-30 min, the subsequent 30-60 min, and the cumulative 0-60 min period of analysis. Saline vehicle infusion alone did not affect chow intake. After i.c.v. saline, restraint stress significantly reduced food intake during the first 30 min. This hypophagic effect did not recover during the second 30 min, such that cumulative 60 min intake remained suppressed after restraint. On its own, i.c.v. Ex9 slightly but significantly increased food intake compared to baseline intake during the first 30 min, but not as compared to intake after i.c.v. saline alone. Intake after Ex9 treatment alone was subsequently reduced during the second 30 min, such that no significant effect remained at the cumulative 60 min time point. Pretreatment with i.c.v. Ex9 significantly attenuated restraint stress-induced hypophagia during the first 30 min and at the cumulative 60 min time point. At all time points, food intake was statistically similar in rats under baseline, i.c.v. saline, and i.c.v. Ex9 + Restraint conditions. *, significantly different ($p < 0.05$) compared to all other treatment groups at the same time point. #, significantly different ($p < 0.05$) compared to baseline and Ex9 + Restraint groups at the same time point. &, significantly different ($p < 0.05$) compared to baseline at the same time point.

3.4 DISCUSSION

Periods of caloric deficit attenuate central drive to the HPA axis, anxiety-like behavior, and stress-induced hypophagia in rats, while concurrently increasing circulating levels of CORT at baseline and in response to stress (Akana et al., 1994; Hanson et al., 1994; Lennie et al., 1995; Youngblood et al., 1997; Heiderstadt et al., 2000; Levay et al., 2007). This metabolic tuning of behavior and physiology may promote foraging and food intake during periods of negative energy balance, while concurrently facilitating the maintenance of glucose-dependent processes (Dallman et al., 1999). Results from the present study reveal a potential central neural mechanism for this shift in stress responsiveness: food deprivation eliminates or markedly reduces the ability of cognitive stress to activate GLP-1 and PrRP/NA neurons within the cNTS. These hindbrain neural populations are known to contribute to anxiety-like behavior, HPA axis activation, and stress-induced hypophagia via axonal projections to brainstem, hypothalamic, and limbic forebrain targets (Rinaman, 1999a; Lawrence et al., 2000; Cecchi et al., 2002; Ciccocioppo et al., 2003; Kinzig et al., 2003; Mera et al., 2006; Choi et al., 2007). Consistent with this, we report that overnight fasting reduces anxiety-like behavior and that central GLP-1 receptor signaling is necessary for cognitive stress-induced hypophagia, reminiscent of previous findings that central GLP-1 signaling contributes to anxiety-like behavior and endocrine responses to stress (Kinzig et al., 2003). Thus, fasting-induced "silencing" of GLP-1 and PrRP/NA neurons offers a potential mechanism through which negative energy balance decreases centrally mediated behavioral responses to stress.

Fasting Decreases GLP-1 cFos Activation

We report that GLP-1 neurons within the cNTS and medullary reticular formation are activated by cognitive stress (i.e., restraint and elevated platform exposure) in rats fed *ad libitum*. Considering that GLP-1 neurons in rats are glutamatergic (Zheng & Rinaman, submitted) and that their axon terminals synapse directly onto CRH neurons within the mpPVN (Sarkar et al., 2003a), stress-induced activation of hindbrain GLP-1 neurons likely drives activation of mpPVN neurons at the apex of the HPA axis. Indeed, GLP-1 receptor signaling facilitates glutamate release and increases spike frequency in PVN neurons (Acuna-Goycolea and Pol, 2004), contributing to increased plasma CORT concentrations (Kinzig et al., 2003).

In marked contrast to the results summarized above in rats fed *ad libitum*, an overnight fast abolished GLP-1 cFos activation under all conditions assessed in the present study, evidence that caloric deficit inhibits GLP-1 neurons, blocks their responses to excitatory synaptic input, and/or silences that input. These results extend a previous report indicating that fasting eliminates GLP-1 cFos activation in response to visceral stress (Maniscalco and Rinaman, 2013). The consequences of abolished GLP-1 neural recruitment could include anxiolysis and blunted activation of mpPVN neurons at the apex of the HPA axis (Kinzig et al., 2003), attenuation of stress-induced hypophagia (present study), and modification of other stress-related responses in which GLP-1 signaling has been implicated, including sympathetic activation, reduced gastric emptying, hyperthemia, and increased colonic motility (O'Shea et al., 1996; Gulpinar et al., 2000; Yamamoto et al., 2002).

While numerous studies have noted the presence of GLP-1 neurons within the medullary reticular formation (Larsen et al., 1997b; Rinaman, 1999b), ours is the first to quantify and report their substantial contribution (i.e., ~44%) to the total GLP-1 population. Our results revealed no

difference between cNTS and reticular GLP-1 neurons in their activation after cognitive stress (Table 4), suggesting that both populations receive similar direct and relayed central inputs from hypothalamic and limbic forebrain regions that convey cognitive stress signals to the caudal medulla (Dayas and Day, 2001; Dayas et al., 2004).

Fasting Decreases Activation of PrRP$^+$ and PrRP$^-$ NA neurons of the A2 cell group

In addition to activating GLP-1 neurons, restraint and elevated platform stress also significantly increased cFos activation of the PrRP$^+$ subpopulation of NA A2 neurons in ad lib-fed rats, with a greater degree of activation induced by restraint (~90%) compared to elevated platform exposure (~ 52%). These results are consistent with previous reports that PrRP$^+$ A2 neurons are activated in rats after cognitive stress, including water-immersion restraint (Maruyama et al., 2001), footshock (Morales and Sawchenko, 2003), and conditioned fear cues (Zhu and Onaka, 2003), and provide the first evidence that they also are activated after 5-min exposure to an elevated platform. Further, we discovered that overnight fasting eliminates cFos activation of PrRP$^+$ A2 neurons under baseline control conditions and after elevated platform exposure, and substantially attenuates their otherwise robust responses to restraint stress. These results indicate that PrRP$^+$ A2 neurons within the cNTS are inhibited or otherwise "silenced" by overnight food deprivation, similar to GLP-1 neurons.

Activation of NA/PrRP A2 neurons likely contributes to stress-induced activation of the HPA axis and to behavioral anxiety, since these neurons project densely to the mpPVN and anterior vlBST (Maruyama et al., 1999; Morales et al., 2000; Banihashemi and Rinaman, 2006; Rinaman, 2010). Central ventricular or intra-PVN administration of norepinephrine (NE) or PrRP drives HPA axis activation (Plotsky, 1987; Itoi et al., 1999; Cole and Sawchenko, 2002;

Seal et al., 2002; Mera et al., 2006), and pharmacological antagonism of NA signaling or immunotoxic lesion of A2 neurons (presumably including the $PrRP^+$ subset) that innervate the mpPVN and anterior vlBST attenuates behavioral anxiety and neuroendocrine responses to stress (Cecchi et al., 2002; Bienkowski and Rinaman, 2008; Zheng and Rinaman, 2013). Considering this, our findings support the view that fasting-induced attenuation of A2 NA/PrRP neuronal signaling plays a key role in attenuating behavioral and physiological responses to acute stress.

Less is known regarding the specific functions of the $PrRP^-$ population of NA neurons within the cNTS. $PrRP^-$ NA neurons displayed negligible cFos activation at baseline, regardless of feeding status. In ad lib-fed rats, cFos activation of $PrRP^-$ NA neurons was increased after both cognitive stressors, with a larger increase after restraint stress compared to elevated platform exposure, similar to the $PrRP^+$ neural population. Notably, however, $PrRP^-$ NA neurons were significantly less stress responsive than $PrRP^+$ or GLP-1 neurons, with a maximal response of only ~25% activation after restraint. This difference in relative stress sensitivity may result from the anatomical location of $PrRP^-$ NA neurons. The more stress-responsive $PrRP^+$ population of A2 neurons and GLP-1 neurons are located caudal to the AP, whereas most $PrRP^-$ NA neurons are located at and rostral to the AP. Comparisons of stress-induced cFos activation of A2 neurons indicates that cognitive stressors recruit A2 neurons at more caudal levels than those recruited by visceral stressors (Dayas et al., 2001b), consistent with a report that visceral stressors do not activate $PrRP^+$ NA neurons as robustly as do cognitive stressors (Morales and Sawchenko, 2003). Despite the lower sensitivity of $PrRP^-$ A2 neurons to cognitive stress, their reduced activation after an overnight fast indicates that they also are sensitive to feeding status.

Mechanisms Underlying Fasting-Mediated Suppression of GLP-1 and A2 Neural Activation

We can only speculate as to how an overnight fast reduces cFos activation within hindbrain GLP-1 and NA A2 neurons. Since rats consume most of their calories during the dark cycle, a single overnight fast generates acute negative energy balance and elicits marked changes in physiology, including activation of the HPA axis during the dark cycle in a pattern that corresponds to the missed meals (Akana et al., 1994; Dallman et al., 1999). Conceivably, this could generate feedback signals that inhibit GLP-1 and A2 neurons, desensitize them to excitatory inputs, and/or silence those inputs during the subsequent morning (i.e., when experiments were conducted in the present study). Neurons within the cNTS receive direct glutamatergic innervation from vagal afferent neurons as well as inputs from neurons in a variety of brainstem, hypothalamic, and limbic forebrain regions, and are also sensitive to circulating factors that convey information regarding digestive and metabolic status (Altschuler et al., 1989; Miselis et al., 1991; Schwartz et al., 1991a; Stornetta et al., 2002; Appleyard et al., 2007; Hisadome et al., 2010; Potes and Lutz, 2010; Maniscalco et al., 2013). Fasting may reduce excitatory inputs or increase inhibitory inputs to GLP-1 and A2 neurons, thereby preventing their activation after cognitive stress. For example, circulating levels of leptin are decreased while levels of the gut hormone ghrelin are increase during fasting (Dallman et al., 1999; Tschop et al., 2000; Pico et al., 2002), and vagal sensory signaling to the cNTS is increased by leptin signaling and decreased by ghrelin signaling (Date et al., 2002; Peters et al., 2004; Date et al., 2005; Peters et al., 2006a; Cui et al., 2011), providing two routes through which fasting-mediated peripheral hormonal changes may modulate responsiveness in hindbrain neurons.

Fasting Decreases Neural Activation in the Anterior vlBST

Neurons within the anterior vlBST exert control over autonomic, neuroendocrine, and behavioral responses to homeostatic threats via efferent projections to the mpPVN, central nucleus of the amygdala, periaqueductal gray, and numerous other brain regions (Gray and Magnuson, 1992; Dong et al., 2001; Walker et al., 2003; Crestani et al., 2013). Lesions or pharmacological inactivation of neurons within the vlBST decrease anxiety-like behavior (Walker and Davis, 1997), decrease basal mpPVN CRH mRNA expression (Herman et al., 1994) and attenuate stress-induced increases in mpPVN cFos activation, plasma ACTH levels, and plasma CORT concentrations (Choi et al., 2007). The anterior vlBST – particularly the fusiform subnucleus – receives moderate innervation by GLP-1 neurons (Rinaman, 2010) and particularly dense innervation from $PrRP^{+}$ A2 neurons (Forray and Gysling, 2004; Banihashemi and Rinaman, 2006) (Figure 9). Further, neuroendocrine and anxiety-like responses to cognitive stress are mimicked and facilitated by central PrRP and NA signaling, including NA signaling targeted to the anterior vlBST (Cecchi et al., 2002; Cole and Sawchenko, 2002; Mera et al., 2006). Considering this, we hypothesized that fasting-induced "silencing" of GLP-1 and $PrRP^{+}$ A2 neurons would attenuate stress-induced activation of both mpPVN and anterior vlBST neurons. Although a main effect of feeding status on mpPVN cFos activation failed to reach statistical significance (p = 0.07), stress-induced activation of neurons within the anterior vlBST was significantly attenuated in fasted rats, consistent with fasting-induced suppression of excitatory inputs to the anterior vlBST that arise from GLP-1 and $PrRP^{+}$ A2 neurons (Cecchi et al., 2002; Stornetta et al., 2002; Banihashemi and Rinaman, 2006)(Zheng and Rinaman, submitted).

Fasting Decreases Anxiety-like Behavior

Acute overnight fasting significantly reduced anxiety-like behavior on the EPMZ, consistent with decreased cFos expression within the anterior vlBST and within hindbrain $PrRP^{+}$ A2 and GLP-1 neurons. Ad lib-fed rats spent a majority of the 5 min test moving between the two closed arms, with very little open arm exploration. Fasted rats, however, displayed significantly increased open arm exploration, as evidenced by increased open arm time and entries compared to ad lib-fed rats. Our results are consistent with the hypothesis that the anxiolytic effects of caloric deficit permit increased exploration of the "anxiogenic" environment, i.e., the brightly lit open arms of the maze. There were no differences, however, between fasted and fed rats in the total number of arm entries or total distance traveled on the maze during the 5 min test, arguing against a general increase in locomotion after fasting. These results are consistent with the hypothesis that fasting-induced suppression of GLP-1 and/or NA/PrRP signaling to the anterior vlBST contributes to the anxiolytic effects of caloric deficit.

Fasting Increases CORT levels at Baseline and After Cognitive Stress

Fasting elevated baseline plasma CORT levels, consistent with some (Kiss et al., 1994) but not all (Akana et al., 1994) previous reports. Regardless of feeding status, CORT levels after elevated platform exposure were lower than levels after restraint stress, evidence that restraint is the more "stressful" treatment. In ad lib-fed rats exposed to either stressor, CORT levels were significantly elevated 30 min after stress onset and markedly reduced 30 min after stress termination, consistent with previous reports (Bhatnagar and Dallman, 1998). Fasting, however, significantly increased peak CORT levels and delayed recovery after both stressors, particularly following restraint. These results validate previous reports that fasting increases peak CORT

levels after restraint stress (Akana et al., 1994; Kiss et al., 1994), and newly demonstrate that fasting also interferes with post-stress recovery of baseline CORT levels. CORT synthesis occurs within minutes of stressor onset (Sapolsky et al., 2000), and fasting has been reported to reduce plasma CORT degradation and clearance in rats (Herbst et al., 1960; Woodward et al., 1991; Kiss et al., 1994). Thus, even though fasting appears to reduce central drive to the HPA stress axis (i.e., to CRH neurons within the mpPVN), reduced CORT catabolism might explain the higher CORT levels measured at baseline and after stress in fasted rats.

Central GLP-1 Signaling is Necessary for Cognitive Stress-Induced Hypophagia

Central GLP-1 signaling has well-established hypophagic properties (Turton et al., 1996; Rinaman, 1999a), and previous reports using the GLP-1 receptor antagonist Ex9 indicate that central GLP-1 signaling is critical for the suppression of food intake by visceral stressors (i.e., lithium chloride and lipopolysaccharide) (Rinaman, 1999a; Grill et al., 2004). The present study provides the first evidence that central GLP-1 signaling also is necessary for the hypophagic effect of cognitive stress (i.e., restraint). Under baseline conditions, rats ate slightly less than 2% of their body weight in chow during the first hour of the dark period, with two-thirds of that intake occurring within the first 30 minutes. This intake pattern was not altered by i.c.v. administration of saline alone. However, i.c.v. saline followed by restraint significantly reduced intake by 70% during the first 30 min, and by 55% overall during the first hour. This finding is consistent with previous reports that restraint stress has anorectic effects (Krahn et al., 1986; Rybkin et al., 1997). The most important finding of our experiment was that the hypophagic response to restraint was abolished by i.c.v. pretreatment with Ex9, strongly supporting the hypothesis that endogenous GLP-1 receptor signaling contributes to (or mediates) cognitive

stress-induced hypophagia. Although we did not localize the central site(s) of action at which GLP-1 signaling suppresses food intake after restraint stress, a previous report indicates that 4th ventricular Ex9 attenuates hypophagia in rats after lipopolysaccharide administration (Grill et al., 2004). Thus, a brainstem site of action appears likely. Although central PrRP signaling also suppresses food intake and may contribute to stress-induced hypophagia (Lawrence et al., 2000; Maniscalco et al., 2013), we did not evaluate this possibility given the current lack of pharmacological tools to antagonize PrRP receptors.

Conclusions

Results from the present study indicate that a single overnight fast markedly reduces the ability of cognitive stress to activate GLP-1 and PrRP^{+} A2 neurons within the cNTS. Fasting also reduces neuronal activation within the NA/PrRP terminal-rich vlBST, and decreases anxiety-like behavior. Our results also establish a critical role for endogenous GLP-1 receptor signaling in cognitive stress-induced hypophagia. We conclude that metabolic tuning of hindbrain GLP-1 and PrRP^{+} A2 neurons is a potential mechanism through which negative energy balance decreases central drive to the HPA axis, promotes anxiolysis, and attenuates hypophagic responses to acute stress.

4.0 GHRELIN RECEPTOR SIGNALING CONTRIBUTES TO FASTING-INDUCED SUPPRESSION OF HINDBRAIN NEURAL RESPONSES TO SYSTEMIC CHOLECYSTOKININ

4.1 INTRODUCTION

We recently reported that an overnight fast eliminates or markedly attenuates the ability of systemically administered cholecystokinin-8 (CCK) to activate cFos expression within hindbrain glucagon-like peptide-1 (GLP-1) neurons and noradrenergic (NA) A2 neurons that co-express prolactin-releasing peptide (PrRP) (Maruyama et al., 2001; Maniscalco and Rinaman, 2013). In *ad libitum* (ad lib)-fed rats, CCK increases the firing rate of gastrointestinal vagal sensory neurons that innervate the caudal (visceral) nucleus of the solitary tract (cNTS), activating GLP-1, $PrRP^+$ A2, and other cNTS neurons, thereby suppressing food intake (Smith et al., 1981; Raybould et al., 1988; Luckman, 1992; Schwartz et al., 1993; Monnikes et al., 1997; Rinaman, 1999b; Lawrence et al., 2002; Rinaman, 2003a; Appleyard et al., 2007). In addition to eliciting satiety/hypophagia (Lawrence et al., 2000; Hayes et al., 2009), central GLP-1 and NA/PrRP signaling is strongly implicated in numerous physiological and behavioral components of energy balance and stress responses (Matsumoto et al., 2000; Cecchi et al., 2002; Kinzig et al., 2003; Maniscalco et al., 2013). Thus, the ability of food deprivation to decrease activation of GLP-1 and $PrRP^+$ A2 neurons may contribute to attenuations observed in both satiety

signaling/hypophagia and centrally mediated stress responses during periods of caloric deficit (Akana et al., 1994; Hanson et al., 1994; Lennie et al., 1995; Genn et al., 2003; Inoue et al., 2004) (Chapter 3). Overnight fasting in rats, however, produces widespread metabolic and physical changes (Dallman et al., 1999), many of which might directly or indirectly affect hindbrain GLP-1 and $PrRP^{+}$ A2 neurons. Identifying which components of fasting are responsible for altering activation of these neurons in response to systemic CCK will therefore clarify how satiety signaling and neural stress responses are reorganized during periods of negative energy balance.

In fasted rats, reduced responsiveness of GLP-1 and $PrRP^{+}$ A2 neurons to systemic CCK treatment could result from the loss of an excitatory signal that is present in ad lib-fed rats, the generation of an inhibitory signal associated with food deprivation, or both. Since CCK-induced activation of cNTS neurons depends on the integrity of glutamatergic vagal afferent signaling (Raybould et al., 1988; Monnikes et al., 1997; Appleyard et al., 2007), fasting-induced modulation of GLP-1 and $PrRP^{+}$ A2 neuronal response to CCK could occur at the level of vagal afferents and/or within the cNTS itself. Considering this, we speculated that fasting-induced changes in leptin and/or ghrelin signaling are involved, as circulating levels of leptin and ghrelin reflect energy balance, and each hormone is poised to signal at vagal sensory neurons and within the cNTS.

Leptin, the peptide product of the *obese (Ob)* gene, is released from adipose tissue and the gastric epithelium to signal long- and short-term caloric surfeit, respectively (Zhang et al., 1994; Bado et al., 1998). Peripherally, leptin binds receptors on the vagus nerve (Buyse et al., 2001; Burdyga et al., 2002) and depolarizes vagal afferents that synapse within the cNTS (Peters et al., 2006a). Following transport through the blood brain barrier (BBB) (Banks et al., 1996),

leptin acts directly on neurons of the cNTS, including $PrRP^+$ A2 neurons (Hay-Schmidt et al., 2001; Ellacott et al., 2002; Maniscalco and Rinaman, 2014). Since plasma leptin levels fall markedly in rats during overnight fasting (Dallman et al., 1999; Pico et al., 2002), reduced leptin signaling is a strong candidate factor underlying fasting-induced decreases in GLP-1 and $PrRP^+$ A2 neuronal responses to exogenous CCK. Indeed, a substantial literature indicates that leptin signaling facilitates CCK-induced activation of cNTS neurons. Central leptin administration increases CCK-induced cFos expression within the cNTS (Emond et al., 1999) and A2 neurons (Williams et al., 2009), an effect that is recapitulated following peripheral leptin administration (Wang et al., 1998). The latter effect likely results, at least in part, from direct leptin signaling on vagal sensory neurons, since the majority of vagal afferents that are depolarized by leptin are also depolarized by CCK *in vitro* (Peters et al., 2006b), and co-administration of leptin enhances CCK-induced elevations in cytosolic calcium within cultured nodose neurons (Peters et al., 2004). Thus, marked reductions in CCK-induced activation of GLP-1 and A2 neurons in fasted rats could result from reduced leptin signaling at the level of the vagus nerve, the cNTS, or both.

Ghrelin is a potent orexigenic hormone produced by cells of the upper gastrointestinal (GI) tract (Kojima et al., 1999; Date et al., 2000; Toshinai et al., 2001). Unlike leptin and most GI hormones, plasma levels of ghrelin rise during fasting (Tschop et al., 2000; Bagnasco et al., 2002), and a growing body of literature implicates ghrelin signaling in the suppression of vagally-mediated responses to CCK. Following release from the stomach, ghrelin acts at peripheral receptors expressed by CCK-sensitive vagal afferents to decrease their firing rate (Date et al., 2002; Date et al., 2005). Moreover, ghrelin pre-treatment prevents CCK-induced increases in vagal afferent spike frequency (Date et al., 2005), evidence that ghrelin signaling not only inhibits activation of vagal afferents and subsequent glutamate release in the cNTS

(Appleyard et al., 2007), but also prevents the otherwise robust activation of these afferents by CCK. Similar to leptin, ghrelin has direct central effects following transport across the BBB (Banks et al., 2002), the physiological importance of which is highlighted by the fact that ghrelin transport is facilitated during fasting (Banks et al., 2008). Within the cNTS, ghrelin acts directly on the synaptic terminals of vagal afferents to suppress glutamate release onto A2 cNTS neurons, resulting in decreased A2 firing rate (Cui et al., 2011). Interestingly, the ability of ghrelin to suppress glutamatergic vagal afferent signaling is enhanced in rats after an 18 hr overnight fast (Cui et al., 2011), suggesting that ghrelin's signaling properties are more potent in fasted animals. Together, these findings strongly suggest that increased ghrelin signaling during periods of caloric deficit actively suppresses the ability of CCK to recruit hindbrain GLP-1 and $PrRP^{+}$ A2 neurons.

Considering the evidence presented above, we hypothesized that experimental manipulations to either increase circulating levels of leptin or to block ghrelin receptor signaling in fasted rats would rescue the ability of systemically administered CCK to activate GLP-1 and $PrRP^{+}$ A2 neurons within the cNTS.

4.2 MATERIALS & METHODS

Animals

Adult male Sprague-Dawley rats (Harlan, IN; 225-275g BW) were housed singly in hanging stainless steel wire mesh cages in a temperature-controlled room (20-22°C) on a 12/12 hr light/dark cycle (lights on at 0700 hr). Rats had *ad libitum* access to pelleted chow (Purina 5001) and water, except as noted. All 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. Rats were weighed one day prior to systemic drug treatments to determine proper dosage. Rats were returned to their home cage and deprived of food (but not water) for 16-18 hr overnight before injection.

Experiment 1: Leptin co-administration with CCK

On the day of the experiment rats were removed from their home cage between 0830 and 1030 hr and injected intraperitoneally (i.p.) with 1.0 ml of sterile 0.15 M NaCl containing leptin alone (400 or 800 μg/kg BW; Sigma-Aldrich; L5037; n =4) or leptin plus sulfated CCK (3 μg/kg BW; Bachem; H-2080; n = 4). CCK and/or leptin were dissolved in vehicle just prior to injection, and rats were returned to their home cage immediately after injection. The 3 μg/kg BW dose of CCK elicits robust cFos activation in GLP-1 and A2 neurons in ad lib-fed rats, which is significantly reduced in fasted rats (Maniscalco and Rinaman, 2013). The 400 and 800 μg/kg BW doses of leptin each elicit a maximal pSTAT3 response in the cNTS in fasted rats (Maniscalco and Rinaman, 2014). Since there were no differences in the ability of the two leptin doses to alter hindbrain neural activation in the present study, data from rats receiving either dose were combined for statistical purposes.

Experiment 2: Ghrelin antagonist pretreatment and CCK

On the day of the experiment, rats (n = 20) were removed from their home cage between 0830 and 1030 hr and received one of the following four i.p. treatments: 1) vehicle (0.15M NaCl), 2) vehicle containing ghrelin receptor antagonist (GRA; [D-Lys^3]-GHRP-6; 3.3 mg/kg BW; Sigma-Aldrich, G4535), 3) vehicle followed 30 min later by CCK (3 μg/kg BW), or 4) GRA followed

30 min later by CCK (n = 4-8 per treatment group). Pretreatment with GRA (or saline, to control for dual injections) was performed in order to achieve ghrelin receptor blockade before CCK treatment, and published data indicate that GRA robustly suppresses food intake in rats within 30 minutes after i.p. administration (Asakawa et al., 2003).

Perfusions & immunohistochemistry

Ninety minutes after the first (or only) injection, rats were deeply anesthetized with pentobarbital sodium (39 mg/1.0 ml i.p., Fatal Plus Solution; Butler Schein) and perfused transcardially with a brief saline rinse followed by fixative (100 ml of 2% paraformaldehyde and 1.5% acrolein in 0.1M phosphate buffer, followed by 100 ml of 2% paraformaldehyde alone). Brains were post-fixed *in situ* overnight at 4°C, then removed from the skull and cryoprotected for 24-48 hr in 20% sucrose. Brains were blocked and sectioned coronally (35μm) using a Leica freezing-stage sliding microtome. Tissue sections were collected in six serial sets, and stored at -20°C in cryopreservant solution (Watson et al., 1986) until immunohistochemical processing. Tissue sets from rats injected i.p. with CCK (3 μg/kg BW) and processed according to the same injection and perfusion protocol used here (n = 4 ad lib-fed; n = 4 DEP), were obtained from a recent study (Maniscalco and Rinaman, 2013). These tissue sets were processed for immunohistochemistry together with tissue sets from animals here, providing comparisons to treatment groups in experiments 1 and 2.

Primary and secondary antisera were diluted in 0.1M phosphate buffer containing 0.3% Triton X-100 and 1% normal donkey serum. Two sets of tissue sections from each rat were incubated in a rabbit polyclonal antiserum against cFos (1:20,000; EMD Chemicals, PC38), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch). Sections

were then treated with Elite Vectastain ABC reagents (Vector) and reacted with diaminobenzidine (DAB) intensified with nickel sulfate to produce a blue-black nuclear cFos reaction product. To visualize cFos within hindbrain GLP-1 neurons, one set of cFos-labeled tissue sections was subsequently incubated in a rabbit polyclonal antiserum against GLP-1 (1:10,000; Bachem, T-4363), followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch), Elite Vectastain ABC reagents (Vector), and reacted with plain DAB to produce a brown cytoplasmic reaction product. To visualize cFos within $PrRP^+$ A2 neurons in the cNTS, the second set of cFos-labeled tissue sections was incubated in rabbit anti-PrRP (1:1,000; Phoenix Pharmaceuticals, H-008-52). After rinsing, sections were incubated in Cy3-conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch) to produce a red fluorescent cytoplasmic signal.

Imaging & quantification of cFos expression by GLP-1 neurons

GLP-1 neurons were visualized using a light microscope and 20/40X objectives to determine the number of single-labeled $GLP\text{-}1^+$ neurons, and the number of double-labeled $GLP\text{-}1^+/cFos^+$ neurons. Single- and double-labeled GLP-1 neurons were quantified bilaterally within the cNTS and adjacent reticular formation, and counts were made through the entire rostrocaudal extent of the GLP-1 cell group (i.e., from the cervical spinal cord through the NTS just rostral to the area postrema; ~15.46 mm to 13.15 mm caudal to bregma). Criteria for counting a neuron as $GLP\text{-}1^+$ included clear cytoplasmic labeling and a visible nucleus. Criteria for counting a neuron as double-labeled included brown GLP-1 cytoplasmic labeling and a nucleus that contained visible blue-black cFos immunolabeling, regardless of intensity. In each analyzed case, cFos activated GLP-1 neurons were represented as a percent of total GLP-1 neurons.

Imaging & quantification of cFos expression by PrRP$^+$ A2 neurons

PrRP$^+$ A2 neurons were imaged in the caudal NTS using a 20X objective on an Olympus microscope equipped for brightfield and epifluorescence, and photographed using a digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Neurons were counted in photographic images using Adobe Photoshop CS4 image software. Criteria for counting a neuron as PrRP$^+$ included clear cytoplasmic labeling and a visible nucleus. Neurons were considered cFos$^+$ if their nucleus contained blue/black cFos immunoperoxidase labeling, regardless of intensity. Single- and double-labeled neurons were counted bilaterally through the rostrocaudal extent of the PrRP$^+$ cell group (i.e., from the cervical spinal cord through the NTS at the level of the area postrema; ~15.46 mm to 13.15 mm caudal to bregma). In each analyzed case, cFos activated PrRP$^+$ neurons were represented as a percent of total PrRP$^+$ neurons.

Statistics

Separate one-way ANOVAs were used to determine the effect of leptin co-administration or GRA pretreatment on CCK-induced cFos activation within hindbrain GLP-1 or PrRP$^+$ A2 neurons in fasted rats. When F values indicated a significant main effect of i.p. treatment, the ANOVA was followed by Fisher's least significant difference post hoc analyses. Differences were considered significant when $p < 0.05$.

4.3 RESULTS

Experiment 1: Leptin co-administration with CCK

Leptin co-administration did not increase the ability of CCK to activate cFos in hindbrain GLP-1 or $PrRP^+$ A2 neurons in fasted rats (Figure 13). ANOVA indicated a significant effect of treatment on the proportion of GLP-1 and $PrRP^+$ A2 neurons that were activated to express cFos [GLP-1: $F(3,12) = 153.173$, $p < .001$; PrRP: $F(3,12) = 63.683$, $p < .001$]. Post hoc analyses for each ANOVA indicated that ad lib-fed rats treated with CCK displayed significantly higher proportions of cFos activated GLP-1 and $PrRP^+$ A2 neurons compared to all other treatment groups. There were no other differences in GLP-1 or $PrRP^+$ A2 neural activation between treatment groups.

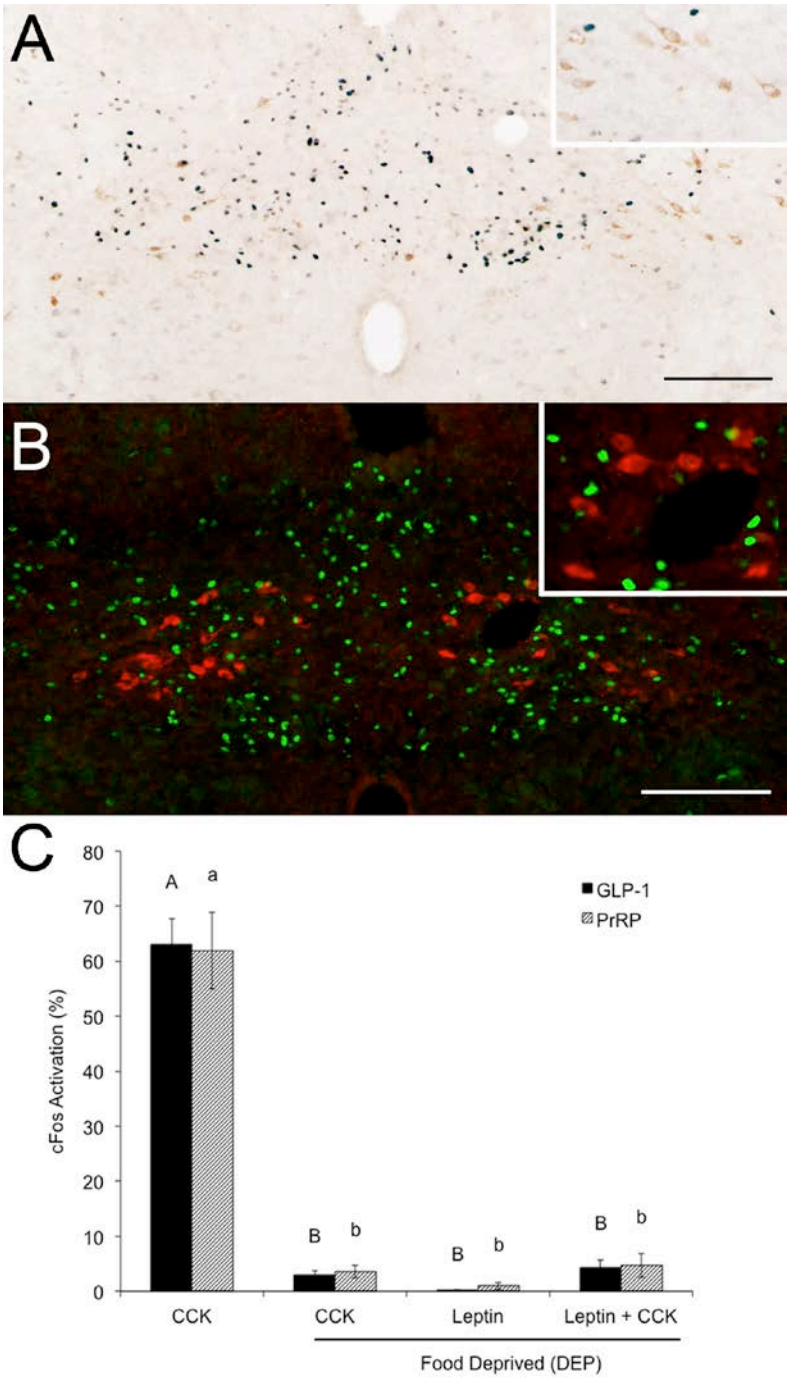

Figure 13. Leptin is not sufficient to rescue CCK-induced GLP-1 or PrRP^{+} A2 cFos activation in fasted rats

A, color image depicting neuronal cFos expression (black nuclear label) and GLP-1^{+} neurons (brown cytoplasmic label) in the cNTS of a rat that received i.p. co-injection of CCK (3 μg/kg BW) and leptin (800 μg/kg BW). Despite

robust cNTS cFos activation, very few $cFos^{+}/GLP\text{-}1^{+}$ neurons are present. *Inset*, higher magnification view of several $GLP\text{-}1^{+}$ neurons, none of which express cFos. B, color image depicting neuronal cFos expression (green nuclear label) and $PrRP^{+}$ neurons (red cytoplasmic label) in the cNTS of a rat that received i.p. co-injection of CCK (3 μg/kg BW) and leptin (800 μg/kg BW). Despite robust cNTS cFos activation, very few $cFos^{+}/PrRP^{+}$ neurons are present. *Inset*, higher magnification view of several $PrRP^{+}$ neurons, none of which express cFos. C, summary data illustrating the proportion of $cFos^{+}$ hindbrain GLP-1 neurons (solid bars) or $PrRP^{+}$ A2 neurons (striped bars). Within the same neuronal population (i.e., GLP-1 or PrRP), bar values with different letters are significantly different ($p < .05$).

Experiment 2: Ghrelin receptor antagonist pre-treatment and CCK

GRA pretreatment in fasted rats partially rescued the ability of CCK to activate cFos expression in both GLP-1 and $PrRP^+$ A2 neurons (Figure 14). ANOVA indicated a significant effect of treatment on the proportion of GLP-1 and $PrRP^+$ A2 neurons that were $cFos^+$ [GLP-1: $F(5,22) = 9.025$, $p < 0.001$; PrRP: $F(5,22) = 8.092$, $p < 0.001$]. Post hoc analyses for each ANOVA indicated that ad lib-fed rats treated with CCK displayed a significantly higher proportion of GLP-1 and $PrRP^+$ A2 neural activation, as compared to all other treatment groups. Additionally, fasted rats receiving GRA + CCK displayed significantly increased activation of GLP-1 and $PrRP^+$ A2 neurons compared to fasted rats receiving CCK alone, saline vehicle alone, GRA alone, or vehicle + CCK. GLP-1 and $PrRP^+$ A2 neural activation was statistically similar between fasted rats receiving CCK alone, saline vehicle alone, GRA alone, or Sal + CCK.

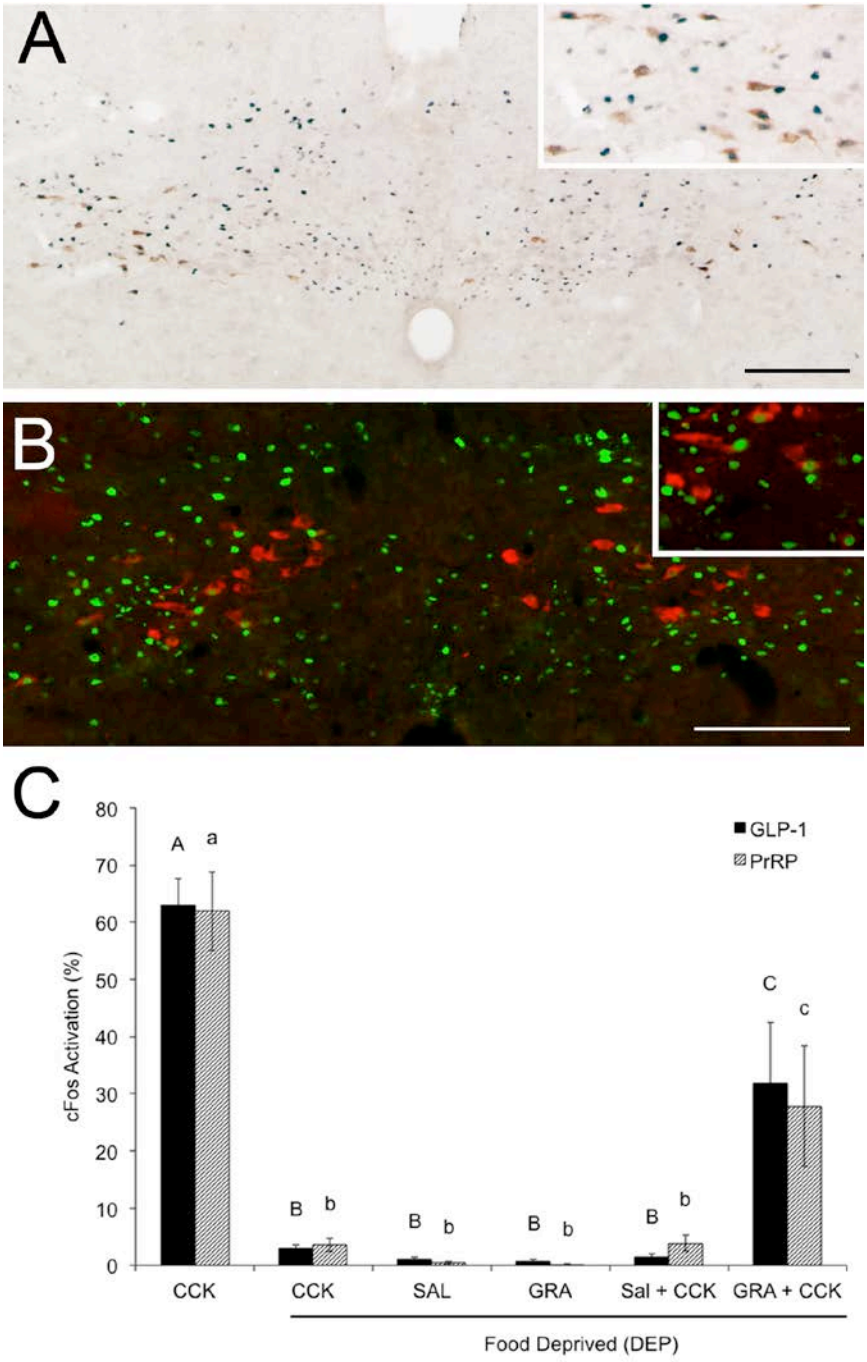

Figure 14. Ghrelin receptor antagonism partially rescues CCK-induced cFos activation of GLP-1 and PrRP$^+$ A2 neurons in fasted rats

Ghrelin receptor antagonist pretreatment partially rescues CCK-induced cFos activation of hindbrain GLP-1 and PrRP$^+$ A2 neurons in fasted rats. A, color image depicting neuronal cFos expression (black nuclear label) within

GLP-1$^+$ neurons (brown cytoplasmic label) in the cNTS of a rat that received i.p. pretreatment with ghrelin receptor antagonist (3.3 mg/kg BW) prior to i.p. CCK (3 μg/kg BW). *Inset*, higher magnification view of several cFos$^+$/GLP-1$^+$ neurons. B, color image depicting neuronal cFos expression (green nuclear label) within PrRP$^+$ neurons (red cytoplasmic label) in the cNTS of a rat that received i.p. pretreatment with ghrelin receptor antagonist (3.3 mg/kg BW) prior to i.p. CCK (3 μg/kg BW). *Inset*, higher magnification view of several PrRP$^+$ neurons, some of which express cFos. C, summary data illustrating the proportion of cFos$^+$ hindbrain GLP-1 neurons (solid bars) or PrRP$^+$ A2 neurons (lined bars). Within the same neuronal population (i.e., GLP-1 or PrRP), bar values with different letters are significantly different ($p < .05$).

4.4 DISCUSSION

Overnight fasting substantially reduces or eliminates the ability of CCK to activate cFos expression within hindbrain A2 NA neurons and GLP-1 neurons (Maniscalco and Rinaman, 2013). We also recently discovered that overnight fasting similarly blocks or attenuates the ability of cognitive stress to elicit cFos activation of GLP-1 and $PrRP^+$ A2 neurons (Chapter 3). However, the mechanisms by which fasting tunes hindbrain neural sensitivity to vagal sensory and other neural inputs are unresolved. Here, we provide evidence that increased ghrelin signaling during overnight food deprivation suppresses CCK-induced activation of both GLP-1 and $PrRP^+$ A2 neurons. Conversely, leptin administration was insufficient to rescue neural cFos responses to CCK in fasted rats.

Fasting decreases GLP-1 and $PrRP^+$ A2 neural cFos activation

Administration of 3 μg/kg BW CCK to ad lib-fed rats robustly activated cFos expression in hindbrain GLP-1 neurons and $PrRP^+$ A2 neurons, consistent with previous reports (Rinaman, 1999b; Lawrence et al., 2002; Maniscalco and Rinaman, 2013). Lawrence et al. (2002) reported that a high dose of CCK (i.e., 50 μg/kg BW) elicits ~85% activation of cNTS $PrRP^+$ A2 neurons in fed rats, whereas an even higher dose (100 μg/kg BW) activated ~57% of GLP-1 neurons in fed rats (Rinaman, 1999b). While the much lower dose of CCK used in the present study (i.e., 3 μg/kg BW) should more closely approximate levels resulting from postprandial release of endogenous CCK, it was sufficient to activate ~60% of GLP-1 and $PrRP^+$ A2 neurons in ad lib-fed rats, likely reflecting the importance of signaling from these neurons in satiety and stress

induced hypophagia (Rinaman, 1999a; Lawrence et al., 2002; Grill et al., 2004; Maniscalco et al., 2013).

In contrast to the robust CCK-induced cFos activation of GLP-1 neurons in ad lib-fed rats, this population displayed little or no cFos response to CCK in fasted rats, consistent with our previous report (Maniscalco and Rinaman, 2013). Although GLP-1 neurons appear to be highly desensitized to stimulus input during fasting, they are not completely refractory, as the strong stimulus of a gastric balloon distention is sufficient to increase activation of these neurons in fasted rats (Vrang et al., 2003). Similar to the GLP-1 population, fasting nearly eliminated CCK-induced activation of $PrRP^+$ A2 neurons, a novel result indicating substantial metabolic sensitivity in the $PrRP^+$ subset of A2 neurons. This finding is particularly interesting when interpreted in light of a previous report that quantified cFos activation in all A2 neurons (i.e., both $PrRP^+$ and $PrRP^-$ subsets) of fed and fasted rats. In that study, a substantial proportion of total A2 neurons was activated by CCK in fasted rats (Maniscalco and Rinaman, 2013). Considering that nearly all $PrRP^+$ A2 neurons are refractory to CCK-induced cFos activation during fasting (Figure 13), activation of the total A2 population during fasting must result nearly entirely from recruitment of $PrRP^-$ neurons. While this has yet to be determined empirically, it suggests a dichotomy in the metabolic sensitivity of $PrRP^+$ and $PrRP^-$ A2 neural subsets, the importance of which is currently unknown.

Ghrelin receptor antagonism partially rescues CCK-induced activation of GLP-1 and $PrRP^+$ A2 neurons in fasted rats

CCK-mediated recruitment of cNTS neurons occurs via activation of glutamatergic vagal afferent fibers (Raybould et al., 1988; Monnikes et al., 1997; Appleyard et al., 2007), and ghrelin

signaling inhibits vagal afferent activation and central vagal glutamate release (Date et al., 2002; Cui et al., 2011). Since circulating ghrelin levels rise during fasting (Tschop et al., 2000), we hypothesized that ghrelin signaling inhibits vagal afferent input to cNTS neurons during an overnight fast, thereby preventing CCK-induced activation of GLP-1 and $PrRP^{+}$ A2 neurons. Indeed, ghrelin receptor antagonism in fasted rats partially rescued CCK-induced cFos activation in GLP-1 and $PrRP^{+}$ A2 neurons, confirming our hypothesis. On average, GRA pretreatment rescued slightly less than 50% of the cFos activation observed in ad lib-fed rats. In fasted rats, no change in cFos activation occurred following saline treatment alone or saline pretreatment prior to CCK, indicating that the handling and stress associated with each injection procedure does not alter cFos activation of these hindbrain neurons. Importantly, GRA administration alone was also not sufficient to increase cFos activation in either neural population, indicating that antagonism of ghrelin signaling permits stimulus-induced activation of GLP-1 and $PrRP^{+}$ A2 neurons without activating the neurons per se. Together, this evidence strongly implicates ghrelin signaling in the metabolic tuning of GLP-1 and $PrRP^{+}$ A2 neurons. As GRA was administered systemically, we are unable to identify its specific site of action; however, it is possible that antagonism of ghrelin receptors influenced vagal afferent signaling at both peripheral (Date et al., 2002) and central (Cui et al., 2011) sites.

Despite producing a significant increase in CCK-induced cFos activation in hindbrain GLP-1 and $PrRP^{+}$ A2 neurons, GRA pretreatment did not fully restore cFos activation to levels observed in ad lib-fed rats. It is possible that the 30-minute latency between pretreatment and CCK administration did not provide sufficient time for GRA to reach target receptors. This time point was chosen based on previous work showing that i.p. GRA administration markedly reduces food intake within 30 minutes (Asakawa et al., 2003). However, ghrelin's hyperphagic

effects occur – at least in part – via the hypothalamic arcuate nucleus (Andrews, 2011). Thus, it is possible that GRA's effects within the arcuate nucleus (and subsequently on food intake) occur more rapidly than GRA's effects on brainstem/vagal afferent neurons. If so, a longer latency between pretreatment and CCK injection may enhance the efficacy of GRA at vagal afferent/brainstem receptors. It is also possible that the suppressive effects of ghrelin signaling on hindbrain neurons occur in conjunction with increases in another inhibitory signal or decreases in an excitatory signal, resulting in a partial (but not full) rescue of neural cFos activation when ghrelin signaling is manipulated in isolation.

Leptin co-administration is insufficient to rescue CCK-induced activation of hindbrain GLP-1 & PrRP$^+$ A2 neurons in fasted rats

A growing body of histological (Wang et al., 1998; Williams et al., 2009) and electrophysiological evidence (Peters et al., 2004; Peters et al., 2006a) indicates that both central and peripheral leptin signaling enhance the ability of CCK to activate neurons within the cNTS. As leptin levels fall markedly during an overnight fast (Dallman et al., 1999), we hypothesized that exogenous leptin (i.p.) in fasted rats would rescue CCK-induced activation of hindbrain GLP-1 and PrRP$^+$ A2 neurons. However, neither leptin administration alone nor leptin co-administration with CCK was sufficient to increase cFos activation of either hindbrain neural population.

It is unlikely that higher doses of systemic leptin are required to alter hindbrain neural sensitivity to CCK, as the doses used here elicit robust pSTAT3 responses within the cNTS (Maniscalco and Rinaman, 2014), and lower doses are sufficient to enhance CCK-mediated transcriptional events in vagal afferents (de Lartigue et al., 2010), decrease food intake

(Campfield et al., 1995; Barrachina et al., 1997; de Lartigue et al., 2010), inhibit gastric emptying (Cakir et al., 2007), and increase sympathetic nervous system activation and lipolysis (Shen et al., 2007). It is possible, however, that pretreatment (versus co-administration) of leptin is necessary to permit CCK-induced activation of GLP-1 and $PrRP^{+}$ A2 neurons in fasted rats. The current study employed a leptin and CCK co-administration strategy based on evidence that leptin directly depolarizes vagal afferent neurons and enhances CCK-mediated calcium influx within seconds of application (Peters et al., 2004; Peters et al., 2006a). Those findings, however, were obtained *in vitro*, and it is possible that the kinetics of leptin diffusion and/or transport following i.p. injection preclude immediate effects on CCK-induced vagal depolarization. In sum, while our results suggest that decreased leptin signaling during fasting does not play a critical role in reducing GLP-1 and $PrRP^{+}$ A2 neural sensitivity to CCK, additional work is needed to strengthen this conclusion.

Conclusion

The results of this study indicate that increased endogenous ghrelin signaling is an important (but not the sole) factor underlying fasting-induced suppression of hindbrain GLP-1 and $PrRP^{+}$ A2 neuronal responses to CCK. By reducing the ability of sensory signals to activate these hindbrain neurons, fasting-mediated increases in ghrelin signaling may alter numerous central processes in which GLP-1 and $PrRP^{+}$ A2 neurons are implicated. For example, A2 neurons (Rinaman, 2003a) and central GLP-1 signaling (Chapter 3) are required for CCK- and restraint-induced suppression of food intake, respectively, and evidence suggests that the ability of stressful stimuli to decrease food intake is attenuated during periods of negative energy balance (Lennie et al., 1995; Youngblood et al., 1997; McMinn et al., 2000). It is tempting to speculate that this may be due,

at least in part, to ghrelin-mediated suppression of $PrRP^{+}$ A2 and GLP-1 neuronal activation. Additional work is needed to examine this possibility.

5.0 GENERAL DISCUSSION

5.1 SUMMARY AND INTERPRETATION OF FINDINGS

5.1.1 Summary

Periods of caloric deficit substantially attenuate many centrally mediated responses to stress, including neural drive to the HPA axis, anxiety-like behavior, and stress-induced suppression of food intake (i.e., stress hypophagia). It is posited that this reorganization of stress responses reduces anxiety and promotes food intake during periods of negative energy balance, increasing the likelihood of finding food and repleting energy stores. The mechanisms by which caloric deficit alters central stress responses, however, remain unclear. Brainstem GLP-1 neurons and $PrRP^{+}$ A2 neurons are stress-sensitive, responsive to changes in the interoceptive environment (including those indicative of food intake and metabolic status), and have been implicated anatomically and functionally in HPA axis activation, anxiety-like behavior, and stress hypophagia. Considering this, we proposed that the ability of caloric deficit to attenuate each of these stress responses depends on reduced signaling from $PrRP^{+}$ A2 neurons and hindbrain GLP-1 neurons. To test this hypothesis, my dissertation research was designed to address 4 overarching aims:

1. Demonstrate the ability of overnight fasting to reduce visceral and cognitive stress-induced activation within hindbrain and forebrain regions integral to HPA axis activation, anxiety-like behavior, and stress hypophagia.
2. Determine whether overnight fasting reduces anxiety-like behavior.
3. Assess the contribution of GLP-1 signaling to cognitive stress-induced hypophagia.
4. Identify specific components of fasting that contribute to reductions in stress-induced neural activation of GLP-1 and $PrRP^{+}$ A2 neurons.

In Chapter 2, we hypothesized that overnight food deprivation would attenuate the ability of CCK, a visceral stressor, to activate cFos within NA A2 and hindbrain GLP-1 neurons as well as mpPVN neurons. This study demonstrated that:

1. In ad lib-fed rats, CCK dose-dependently increased cFos activation within A2 neurons, while the stress of i.p. injection maximally increased GLP-1 neuronal activation.
2. In fasted rats, the ability of i.p. injection and CCK to elicit cFos activation was significantly reduced in A2 neurons and nearly eliminated in GLP-1 neurons, indicating that recruitment of each population is dependent on metabolic state.
3. Overnight fasting attenuated cFos activation in the mpPVN, which correlated significantly with both brainstem GLP-1 and A2 neuronal activation.

Overall, these results support our overarching hypothesis, demonstrating that caloric deficit reduces visceral stimulus-driven recruitment of stress responsive hindbrain and hypothalamic neurons. Therefore, in Chapter 3, we hypothesized that overnight fasting attenuates the ability of

cognitive stress to activate cFos within hindbrain GLP-1 and $PrRP^+$ A2 neurons as well. We also proposed that fasting would attenuate cFos activation within the vlBST and mpPVN – two regions implicated in HPA axis activation and anxiogenesis – in conjunction with decreased anxiety-like behavior. Furthermore, as fasting "uncouples" neurally-mediated stress responses from adrenocortical CORT responses (Akana et al., 1994; Dallman et al., 1999; Genn et al., 2003), we hypothesized that fasting would increase plasma CORT responses to cognitive stressors. Lastly, since cognitive stressors activate brainstem neurons (Section 1.6.1; Chapter 3) and GLP-1 signaling is critical for visceral stress-induced hypophagia (Rinaman, 1999a; Grill et al., 2004), we hypothesized that GLP-1 signaling is also necessary for cognitive stress-induced hypophagia. Our results indicated that:

1. In ad lib-fed rats, cognitive stressors robustly activated GLP-1 and $PrRP^+$ A2 neurons, consistent with previous reports on $PrRP^+$ A2 neurons (Morales and Sawchenko, 2003; Zhu and Onaka, 2003) and providing novel evidence that cognitive stressors recruit hindbrain GLP-1 neurons.
2. Overnight fasting eliminated cognitive stress-induced activation in hindbrain GLP-1 and $PrRP^+$ A2 neurons at baseline and in response to cognitive stress, bolstering our previous report of the metabolic sensitivity of these populations.
3. Overnight fasting decreased anxiety-like behavior and cognitive stress-induced activation of the vlBST, providing novel evidence that overnight food deprivation decreases behavioral anxiety (perhaps via reductions in vlBST output). Fasting-mediated reductions in mpPVN cFos activation approached, but did not reach, significance.

4. Fasting increased plasma CORT levels at baseline and in response to cognitive stress, consistent with previous reports (Akana et al., 1994; Chacon et al., 2005). We newly demonstrate that fasting interferes with post-stress recovery of baseline CORT levels, indicative of a reduction in plasma CORT degradation and clearance (Herbst et al., 1960; Woodward et al., 1991; Kiss et al., 1994).
5. I.c.v. pretreatment with a GLP-1 receptor antagonist abolished RES stress-induced hypophagia in ad lib-fed rats, providing the first evidence that hindbrain GLP-1 neurons are critical for suppression of food intake by cognitive stressors.

In sum, the results of this study support our overarching hypothesis, demonstrating that overnight fasting reduces the ability of cognitive stressors to activate cFos expression within hindbrain and forebrain neurons implicated in HPA axis activation and anxiety. Furthermore, attenuated neural responses occurred in conjunction with decreased anxiety-like behavior and increased plasma CORT levels, substantiating the neural and adrenal "uncoupling" effect documented previously (Akana et al., 1994; Dallman et al., 1999). This study also provides the first evidence that a cognitive stressor requires recruitment of hindbrain GLP-1 neurons to suppress food intake. As GLP-1 neurons appear to be "silenced" at rest and in response to stimulus-driven input during fasting, it is likely that reduced GLP-1 signaling contributes to fasting-mediated reductions in stress hypophagia.

Considering the impact of a single overnight fast on neural, neuroendocrine, and behavioral responses to stress, it was of interest to identify which components of fasting are responsible for reducing activation of hindbrain neurons. To address this, the experiments in Chapter 4 tested the hypothesis that fasting-mediated attenuations in GLP-1 and $PrRP^{+}$ A2 neural

activation in response to CCK result from either decreased leptin signaling, increased ghrelin signaling, or both. Our results indicate that:

1. Systemic leptin co-administration with CCK (i.p.) is insufficient to rescue hindbrain cFos activation in fasted rats.
2. Pretreatment with systemic ghrelin receptor antagonist partially rescues (~50%) CCK-induced cFos activation of both GLP-1 and $PrRP^{+}$ A2 neurons.

The results from this study support the hypothesis that increased ghrelin signaling during an overnight fast plays an integral role in suppressing activation of hindbrain neurons, while challenging the hypothesis that fasting-mediated decreases in plasma leptin attenuate hindbrain neural activation.

5.1.2 Metabolic tuning of neural, neuroendocrine, and behavioral responses to stress: a working model

The results of my dissertation project support a working model in which brainstem neurons integrate peripheral signals of metabolic status into neurally mediated stress responses. Based on our work and the work of others, we propose that:

In an ad lib-fed animal, circulating ghrelin levels are low, permitting tonic and stimulus-induced activation of the vagus nerve and glutamate release within the cNTS. This results in moderate baseline activation of both GLP-1 and $PrRP^{+}$ A2 neurons. Following a visceral stressor (e.g., CCK), vagal afferent firing rate increases and glutamate release drives neural activation within cNTS neurons. Following a cognitive stressor, descending inputs (of unidentified

chemical phenotype) increase neural activation within cNTS neurons. Stress-induced activation of these neurons increases GLP-1, PrRP, NE, and glutamate release at axonal target sites, including the mpPVN, the vlBST, and brainstem reticular neurons. This transmitter signaling (both pre- and postsynaptically) contributes to robust HPA axis activation, anxiety-like behavior, and stress-induced hypophagia (Figure 1).

Conversely, in a fasted animal, circulating ghrelin levels are high, inhibiting tonic and stimulus-induced activation of the vagus nerve and glutamate release within the cNTS, consequently abolishing baseline activation in both GLP-1 and $PrRP^+$ A2 neurons. Increased ghrelin signaling also prevents visceral stress-induced vagal afferent activation and glutamate release, preventing recruitment of cNTS neurons. Following a cognitive stressor, GLP-1 and $PrRP^+$ A2 neural activation is also substantially reduced, possibly due to a direct inhibitory effect of ghrelin on cNTS neurons. Alternatively, it is possible that ghrelin-mediated reductions in tonic vagal afferent activation remove a normally permissive glutamatergic excitatory signal to GLP-1 and $PrRP^+$ A2 neurons, preventing neural activation in response to any input source. Regardless of the exact cause, in the absence of stress-induced activation of these neurons, GLP-1, PrRP, NE, and glutamate are not released at target sites. This reduction of signaling results in reduced drive to the HPA axis, decreased anxiety-like behavior, and attenuation of hypophagic responses to real or anticipated homeostatic threats (Figure 1).

While our findings support this proposed model, it is necessary to address a number of limitations and caveats. First, our results suggest that changes in ghrelin signaling are not the only mechanism by which fasting influences neural stress responses (Chapter 4). It is likely that additional vagally- and non-vagally-mediated signals of metabolic status influence neurons of the cNTS (Sawchenko et al., 2000; Potes and Lutz, 2010). It is also possible that the integration

of metabolic information into central stress signaling is conducted by the cNTS in coordination with other brain regions that are responsive to changes in interoceptive state, including numerous hypothalamic subnuclei (Schwartz et al., 2000; Dhillon et al., 2006). Thus, this model can and should be modified/updated based on the results of future experiments. Second, the ability of fasting-mediated increases in ghrelin signaling to suppress cognitive stress-induced activation of GLP-1 and $PrRP^{+}$ A2 neurons is speculative. Based on our results in rats treated with CCK, however, the impact of systemic GRA on cognitive stress-induced cNTS recruitment should be tested empirically. Finally, and most importantly, while our findings strongly *suggest* that fasting-mediated reductions in GLP-1 and $PrRP^{+}$ A2 neural activation attenuate downstream neural, neuroendocrine, and behavioral stress responses (Chapters 2 & 3), we present no *causal evidence* to support this. It is important that future studies address this (Section 5.3).

5.2 OBSERVATIONS AND CONSIDERATIONS

5.2.1 Additional neurophysiological changes that may contribute to fasting-mediated alterations in central stress responses

Although our work has focused on the contribution of brainstem neurons in fasting-mediated reductions of central stress responses, fasting likely influences additional brain regions that can alter central stress circuits, including the arcuate nucleus of the hypothalamus (ARC). Neurons of the ARC, including those expressing neuropeptide Y/agouti-related protein (NPY/AgRP), are sensitive to changes in metabolic state (Schwartz et al., 2000). Furthermore, overnight fasting increases activation of NPY/AgRP neurons (Yang et al., 2011; Liu et al., 2012) and stimulates

hypothalamic NPY mRNA expression (Hanson et al., 1997; Hahn et al., 1998; Dallman et al., 1999). These results strongly suggest that NPY is released at target sites – including the mpPVN and central extended amygdala (Liposits et al., 1988; Broberger et al., 1998) – during overnight food deprivation. Thus, fasting-mediated increases in NPY signaling could contribute to the changes in food intake, anxiety-like behavior, and HPA axis activation observed in fasted rats.

Indeed, food intake is robustly increased by central administration of NPY (Stanley and Leibowitz, 1984; Dallman et al., 1993) or selective pharmacological/optogenetic stimulation of NPY/AgRP neurons (Aponte et al., 2011; Krashes et al., 2011). Conversely, selective inhibition of these neurons decreases dark-onset food intake (Krashes et al., 2011), and lesions of NPY/AgRP neurons result in marked and persistent decreases in food intake and body weight (Luquet et al., 2005). Thus, in an overnight fasted rat, increased signaling from hypothalamic NPY/AgRP neurons is well suited to complement reductions in activation of satiety-mediating brainstem neurons to elicit both an increased drive for food and larger meals.

Increased NPY signaling may also contribute to the attenuation of behavioral anxiety in fasted rats (Bowers et al., 2012), as central administration or overexpression of NPY mitigates anxiety-like behavior (Heilig et al., 1989; Broqua et al., 1995; Thorsell et al., 2000), while genetic deletion of NPY enhances anxiety-like behavior (Bannon et al., 2000). Furthermore, evidence indicates that the NPY's anxiolytic effects are due – in part – to signaling within the amygdala (Sajdyk et al., 1999), and very likely result from antagonistic effects on anxiogenic CRH signaling within the vlBST (Lee and Davis, 1997; Kash and Winder, 2006).

Although increased NPY signaling is well positioned to drive food intake and reduce anxiety in the fasted state, it does not appear to contribute to reduced neural drive of the HPA axis. In fact, NPY signaling appears to have the converse effect. Within the mpPVN, NPY

signaling stimulates CRH mRNA expression and increases ACTH release (Inoue et al., 1989; Hanson and Dallman, 1995; Seal et al., 2002). Thus, fasting-mediated increases in NPY signaling would be expected to enhance drive to the HPA axis in fasted rats, contrary to previous reports (Akana et al., 1994; Hanson et al., 1994). At this point it is unclear how to reconcile these findings.

Together, these studies indicate that increases in NPY signaling may contribute to fasting-mediated anxiolysis and drive for food intake. It is possible that signaling from brainstem GLP-1 and $PrRP^+$ A2 neurons interacts with and/or influences the NPY system, as GLP-1 and A2 neurons project to the ARC (Rinaman, 2010, 2011) and central GLP-1 administration significantly reduces NPY-stimulated food intake in rats (Turton et al., 1996). Continued anatomical and functional characterization of the effects of caloric deficit on these systems will lead to a more holistic understanding of the impact of metabolic state on neurally mediated stress responses.

5.2.2 Elucidating ghrelin's mechanism of action

In Chapter 4, we demonstrate that ghrelin receptor antagonism partially rescues CCK-induced activation of GLP-1 and $PrRP^+$ A2 neurons in fasted rats (Figure 14). Although the mechanisms by which ghrelin inhibits activation of cNTS neurons during overnight fasting remain unclear, numerous studies implicate ghrelin in the suppression of CCK-induced activation of vagal afferents at either peripheral or central sites. Peripheral ghrelin signaling decreases the spike frequency of vagal afferent neurons (Date et al., 2002) and inhibits CCK-induced increases in vagal afferent activation (Date et al., 2005), while central ghrelin signaling suppresses glutamate release from CCK-sensitive vagal afferent terminals, resulting in decreased postsynaptic spike

frequency of cNTS neurons (Cui et al., 2011). Together, these studies provide strong mechanistic evidence that fasting-mediated increases in ghrelin suppress hindbrain GLP-1 and $PrRP^{+}$ A2 neural cFos activation, in part, via inhibition of vagal afferents.

As vagal afferents innervate many cNTS subnuclei and phenotypically-distinct neural populations (Altschuler et al., 1992), it might be expected that fasting-mediated reductions in vagal afferent activation would attenuate cFos expression in many or all cNTS neural populations. Our qualitative observations, however, indicate that this does not appear to be the case (at least following CCK administration), since robust cFos expression is present within the cNTS of fasted rats despite the complete loss of cFos within GLP-1 neurons (Figure 3). However, quantification of total cFos within the cNTS is necessary to address this possibility. A decrease in total cNTS cFos following CCK administration in fasted rats would support the hypothesis that ghrelin-mediated reductions in vagal glutamatergic input contribute substantially to decreased activation of cNTS neurons. Based on our observations, however, we hypothesize that total cNTS cFos will not be significantly altered, suggesting that ghrelin signaling acts somewhat selectively on the GLP-1 and $PrRP^{+}$ A2 populations, perhaps due to dynamic regulation or selective expression of the ghrelin receptor within these neurons (Lin et al., 2004; Zigman et al., 2006).

Understanding the mechanisms by which ghrelin signaling inhibits cNTS neural activation in response to CCK will provide insight into the potential role of ghrelin signaling in attenuation of cognitive stress-induced activation of GLP-1 and $PrRP^{+}$ A2 neurons. Since cognitive stressors recruit medullary neural activation via descending projections from the forebrain (Figure 1) (Dayas and Day, 2001; Dayas et al., 2004), reductions in vagal afferent input may not influence the ability of these stressors to recruit hindbrain neural activation. However, it

is also possible that excitatory input from vagal afferent neurons is permissive for recruitment of GLP-1 and $PrRP^+$ A2 neurons, regardless of the input source, in which case ghrelin signaling would be expected to influence cognitive stress-induced activation of these neurons as well. Assessing the impact of GRA on total cNTS cFos following CCK as well as the ability of GRA to rescue cFos activation in response to cognitive stressors will provide strong first steps in clarifying ghrelin's influence and mechanisms of action.

5.2.3 Additional factors that may contribute to reduced hindbrain neural activation during fasting

Our observation that GRA only partially rescued CCK-induced activation of GLP-1 and $PrRP^+$ A2 neurons in fasted rats (Chapter 4) suggests that the suppressive effect of ghrelin signaling on hindbrain neurons occurs in conjunction with increases in another inhibitory signal or decreases in an excitatory signal. Although overnight fasting elicits a myriad of physiological changes that might directly or indirectly contribute to attenuation of cNTS cFos activation (Dallman et al., 1999), fasting-mediated changes in circulating glucose, gastric distention, and CORT synthesis are three mechanisms worth considering here.

Although tightly regulated, even during periods of food deprivation, plasma glucose levels fall during an overnight fast (Dallman et al., 1999). Our unpublished observations indicate that plasma glucose levels decrease significantly, from ~90mg/dL in ad lib-fed rats to ~70mg/dL in fasted rats ($p < .05$). Previous studies indicate that cNTS A2 neurons and ventrolateral medulla A1 neurons are glucose sensitive and recruitment of these neurons is necessary for hyperphagic and HPA axis responses to glucoprivation (Ritter et al., 2001; Ritter et al., 2003). Therefore, it is possible that both GLP-1 and $PrRP^+$ A2 neurons are sensitive to blood glucose levels, and that a

decrease in plasma glucose contributes to reductions in GLP-1 and $PrRP^+$ A2 neural activation during fasting. Our preliminary work, however, suggests that this is not the case. The stressor treatments used in Chapters 2-4 (i.e., EP, i.p. saline injection, and i.p. CCK injection) each increase blood glucose levels above those of ad lib-fed rats, presumably as a result of SNS and/or CORT responses to stress (unpublished data). Despite increases in blood glucose, these stressors remain insufficient to increase GLP-1 or $PrRP^+$ A2 neural activation in fasted rats. These observations suggest that the mild drop in blood glucose during fasting does not contribute to changes in hindbrain neural activation. However, glucose homeostasis and signaling is a dynamic and complex process (Watts and Donovan, 2010; Jokiaho et al., 2014), and it is possible that a transient increase in blood glucose resulting from stress exposure has markedly different effects than persistently elevated glucose resulting from ad lib food intake.

Gastric distention is a necessary consequence of food intake and results in rapid and sustained discharges of mechanosensitive vagal afferent nerves (Schwartz et al., 1991a). Thus, gastric distention generates an excitatory (i.e., vagal glutamatergic) signal to cNTS GLP-1 and $PrRP^+$ A2 neurons in ad lib-fed rats, and this signal is likely lost during fasting. Furthermore, as gastric distention and systemic CCK administration have synergistic effects to activate vagal afferents (Schwartz et al., 1991a; Schwartz et al., 1993), reduced gastric distention is poised to have a particularly strong influence on cNTS neural activation in response to systemic CCK (Chapters 2 & 4). Indeed, gastric balloon distention in fasted rats increases cFos activation within A2 neurons (~10% activation) (Willing and Berthoud, 1997) and GLP-1 neurons (~30% activation) (Vrang et al., 2003). While this represents a significant increase as compared to near-0% activation in controls, it is not possible to interpret the degree to which gastric distention "rescued" ad lib-fed baseline neural activation, as this was not assessed. However, in light of our

observations that, in ad lib-fed rats, baseline cFos activation of GLP-1 and $PrRP^+$ A2 neurons is ~30%, reduced gastric distention appears likely to play a major role in fasting-mediated reductions of GLP-1 and $PrRP^+$ A2 neural activation. It is interesting to consider that decreased gastric distention and increased ghrelin signaling may be responsible for a substantial proportion of decreased cNTS neural activation in fasted rats, and an empirical assessment of this possibility is warranted.

During overnight fasting, circulating CORT levels increase significantly in a pattern that corresponds to the missed meals (Akana et al., 1994; Dallman et al., 1999). These large increases are sufficient to allow CORT access to GRs throughout the brain (Spencer et al., 1990), including those expressed by neurons of the NTS (Herman, 1993). As CORT signaling at numerous central sites elicits a "negative feedback" effect on the HPA axis (to prevent excessive neuroendocrine responses) (Herman and Cullinan, 1997), it is possible that overnight elevations in CORT are partially responsible for reduced neural activation in GLP-1 and $PrRP^+$ A2 neurons.

A role for hindbrain GLP-1 neurons in CORT negative feedback has previously been proposed based on evidence that both acute stress and chronic variable stress decrease PPG mRNA in the cNTS (Zhang et al., 2009; Zhang et al., 2010). In these studies, the ability of stress to decrease PPG mRNA was attenuated or abolished by adrenalectomy, and reduced PPG mRNA expression could be recapitulated by acute or chronic exogenous CORT administration. In both cases, reduced PPG mRNA expression was accompanied by reduced immunoreactivity of GLP-1 terminals within the mpPVN. Together, these findings indicate that CORT negative feedback inhibits central GLP-1 synthesis and decreases GLP-1 protein in mpPVN terminals, possibly reducing the ability of GLP-1 to drive neuroendocrine stress responses. More recently, the functional implications of hindbrain CORT negative feedback were directly assessed using cNTS

implantation of micropellets containing CORT or a GR antagonist. cNTS CORT pellets significantly reduced HPA axis responses to acute RES, while GR antagonist pellets increased HPA axis responses to RES as well as anxiety-like behavior on the EPMZ (Ghosal et al., 2014). These results indicate that CORT indeed has negative feedback effects on cNTS neurons, including the GLP-1 subset (Zhang et al., 2009), which result in decreased anxiety-like behavior and HPA axis responses to cognitive stress (Ghosal et al., 2014). The ability of CORT negative feedback to suppress baseline and stimulus-induced cFos activation of hindbrain GLP-1 and $PrRP^{+}$ A2 neurons is not known. However, experiments utilizing adrenalectomy/CORT replacement or CORT micropellet implantation would allow for a clearer understanding of this possibility.

5.2.4 The importance of reticular GLP-1 neurons

A noteworthy result from Chapter 3 is our observation that reticular GLP-1 neurons contribute substantially to the entire GLP-1 population (i.e., ~44% of total GLP-1 neurons). Although numerous studies have noted the presence of GLP-1 neurons within the medullary reticular formation (Larsen et al., 1997b; Rinaman, 1999b), these neurons have been given little consideration. We reported that in ad lib-fed rats reticular GLP-1 neurons are equally as responsive to cognitive stressors as those in the cNTS (Table 4), and a number of important considerations arise from these findings.

First, the afferent inputs activating reticular and cNTS GLP-1 neurons following cognitive stress have not yet been established. Evidence suggests that descending inputs from hypothalamic and limbic forebrain regions convey cognitive stress information to A2 neurons (Dayas and Day, 2001; Dayas et al., 2004), and it seems likely that these inputs drive cognitive

stress-induced cNTS GLP-1 recruitment as well. It is not clear, however, whether these same descending inputs directly innervate reticular GLP-1 neurons, if a parallel set of descending circuits innervates the reticular population, or if reticular GLP-1 neural activation results from relayed signaling via the cNTS.

Second, it is not known whether the axonal projections of reticular GLP-1 neurons are similar to those arising from cNTS GLP-1 neurons. A previous report from our laboratory found complete overlap between brain regions receiving GLP-1 axonal input and those containing fibers labeled with anterograde tracer following injection into the medial cNTS (Rinaman, 2010). Although these results demonstrate that projections from reticular GLP-1 neurons do not innervate regions distinct from projections arising from cNTS neurons as a whole, this study was not able to characterize differences in projections arising from reticular versus cNTS GLP-1 neurons specifically. Therefore, while it appears likely that reticular and cNTS GLP-1 neurons give rise to axons innervating the same target regions, this has not been established empirically, and the possibility remains that reticular GLP-1 axons innervate targets distinct from those innervated by cNTS GLP-1 neurons. Furthermore, even in brain regions that receive innervation from both reticular and cNTS GLP-1 neurons, it is possible that the strength of axonal input from these two populations of GLP-1 neurons differs. Thus, characterizing the axonal topography of reticular versus cNTS GLP-1 neurons, as well as the strength of projections arising from each population within specific target regions, will establish essential anatomical evidence for the homo- or heterogeneity between reticular and cNTS GLP-1 neural populations. This can be achieved through systematic injection of retrograde tracers into GLP-1 terminal plexuses and quantification of the number of cNTS versus reticular GLP-1 neurons projecting to each region. Moreover, by exposing tracer-injected rats to visceral or cognitive stressors prior to sacrifice, it is

possible to identify stress-induced cFos activation of reticular versus cNTS GLP-1 neurons that project to a given target nucleus. These experiments will provide a wealth of functional and anatomical information about brainstem GLP-1 neurons, greatly expanding the results presented in Chapters 2-4.

Third, characterizing cNTS versus reticular GLP-1 neural activation in response to diverse visceral/cognitive stressors will provide clues regarding the functional role of these neurons. Considering the predominant roles of the vagus nerve and cNTS in visceral sensory processing (Section 1.4.1), we hypothesize that many visceral stressors will preferentially recruit cNTS GLP-1 neurons. Consistent with this, unpublished observations from our laboratory indicate that intake of a large meal, a visceral stimulus that recruits vagal afferent signaling (Grill and Hayes, 2012), preferentially activates GLP-1 neurons within the cNTS. Conversely, a recent study demonstrated that i.p. injection of the endotoxin LPS (a visceral stressor) preferentially activates reticular GLP-1 neurons (Gaykema et al., 2009). This is somewhat surprising, as the vagus nerve is poised to transmit signals associated with peripheral LPS exposure (Goehler et al., 1997; Hosoi et al., 2005; Wieczorek and Dunn, 2006), and vagal mediation of this signal would be expected to elicit increased activation within the cNTS (Appleyard et al., 2007). It is possible, however, that the immune response associated with LPS injection elicited activation of reticular neurons by a non-vagal route that involves signaling via endothelial and perivascular cells (Sawchenko et al., 2000). Considering these complexities, a better understanding of the stimuli that activate reticular GLP-1 neurons in the fed and fasted state – as well as investigation of the mechanisms by which these neurons are activated – is warranted.

Lastly, the development of RNA interference to disrupt PPG mRNA expression provides a promising route by which to selectively and chronically manipulate signaling from GLP-1

neurons (Barrera et al., 2011). However, in addition to the challenge of targeting the substantial rostrocaudal distribution of GLP-1 neurons with viral delivery of short-hairpin RNA, it is unlikely that this technique can be used effectively to knockdown PPG expression in the widely-distributed GLP-1 reticular neural population. A major benefit of targeting the cNTS GLP-1 neurons with RNA interference is the ability to explore and identify a selective contribution of these neurons to neural, physiological, and behavioral outcomes. This benefit, however, comes with the significant interpretive challenge associated with negative results, as reticular GLP-1 neurons remain intact.

5.2.5 Understanding the cause of increased circulating CORT during fasting

In Chapter 3, fasting significantly increased peak CORT levels and delayed recovery after both RES and EP. These results validate previous reports that fasting increases peak CORT responses to RES stress (Akana et al., 1994; Kiss et al., 1994), and newly demonstrate that fasting also interferes with post-stress recovery of baseline CORT levels. The mechanism(s) by which fasting augments stress-induced plasma CORT levels in the face of attenuated pituitary ACTH output remain unclear (Akana et al., 1994). Multiple factors, however, might increase adrenocortical sensitivity to ACTH (Bornstein et al., 2008), potentiating stress-induced CORT synthesis despite reduced hypothalamic outflow. For example, stressors increase sympathetic drive to the adrenal gland, which can subsequently increase adrenocortical steroidogenic response to ACTH (Engeland and Gann, 1989; Edwards and Jones, 1993). Thus, it is possible that fasting enhances sympathetic drive to the adrenal gland following cognitive stress, resulting in elevated circulating CORT levels despite reduced ACTH output. The likelihood of this mechanistic explanation, however, is not clear: while removal of adrenal sympathetic innervation reduces

both circadian and dehydration stress-induced increases in plasma CORT levels (Ulrich-Lai and Engeland, 2002; Ulrich-Lai et al., 2006), it does not appear to affect RES stress-induced plasma CORT levels in ad lib-fed rats (Strausbaugh et al., 1999). While this suggests that SNS innervation of the adrenal gland does not impact the magnitude of CORT responses in ad lib-fed rats, it remains possible that adrenal sympathetic innervation is recruited selectively during fasting.

Alternatively, adrenal sensitivity to ACTH and adrenal steroidogenic response to stressors may each remain unchanged during overnight fasting, despite increases in circulating CORT. As CORT synthesis occurs within minutes of stressor onset (Sapolsky et al., 2000) and fasting reduces plasma CORT degradation and clearance in rats (Herbst et al., 1960; Woodward et al., 1991; Kiss et al., 1994), it is possible that reduced CORT catabolism could explain the higher peak CORT levels and reduced post-stress CORT recovery observed in Chapter 3.

5.2.6 Consequences of increased circulating CORT

In Chapter 3, we discussed the likelihood that fasting-mediated elevations in plasma CORT may serve to increase peripheral energy mobilization and utilization, allowing for the maintenance of glucose-dependent processes despite the absence of caloric intake (Dallman et al., 1999; Sapolsky, 2004). It is also possible that increased CORT levels contribute to the stimulation of feeding in fasted rodents. Substantial evidence indicates that increases in glucocorticoid levels are associated with increased food intake, and that administration of glucocorticoids can elicit increases in caloric consumption (Maniam and Morris, 2012), particularly in the presence of low insulin levels (Dallman et al., 1993). Considering that insulin levels quickly fall at the outset of an overnight fast, and remain significantly decreased through the following morning (Dallman et

al., 1999), this evidence implicates elevated glucocorticoid signaling in the drive for food intake during caloric deficit. Finally, increased CORT levels during fasting may have direct or indirect negative feedback effects to suppress central stress responses that produce consequences that are "maladaptive" in the fasted state (i.e., anxiety-like behavior and stress-induced hypophagia). Recent evidence indicates that this type of CORT negative feedback may involve direct GR signaling in the cNTS (Section 5.2.3).

5.2.7 Using ACTH as a measure of hypothalamic outflow

In Chapters 2 & 3, we utilized cFos expression within the mpPVN to understand the strength of neural drive to the HPA axis. While this provides a useful quantitative measure of neural activation (and a measure that can be directly correlated with changes in cFos activation within other brain regions), this method has less quantitative resolution than other available techniques. A more accurate estimation of HPA axis activity would result from identification of cFos expression selectively within mpPVN CRH^{+} neurons, as these neurons are directly responsible for HPA axis activation (Section 1.2). However, in our hands, technical challenges have prevented immunolabeling of a sufficient number of mpPVN CRH neurons to allow accurate quantification. Measurement of plasma ACTH levels arguably represents the most quantitatively rigorous index of "hypothalamic outflow," as circulating ACTH levels are dictated by neural drive to the HPA axis and directly stimulate CORT synthesis in the adrenal cortex (Section 1.2; Figure 1). Furthermore, the use of radioimmunoassay or enzyme immunoassay allows for quantification of the absolute concentration of ACTH in the blood, obviating much of the subjectivity inherent in cFos quantification and more readily allowing for direct comparison of results between studies. As an understanding of the metabolic regulation of HPA axis activation

has become a predominant focus of our work, ensuring that we are using adequately sensitive and quantitatively rigorous measures is imperative.

5.2.8 Fasting does not decrease the number of immunoreactive PrRP neurons

In addition to their role in centrally mediated stress responses, $PrRP^{+}$ A2 neurons have been implicated in satiation and the maintenance of energy homeostasis (Section 1.6.4) (Onaka et al., 2010; Maniscalco et al., 2013). Interestingly, results from previous studies indicate that periods of negative energy balance, such as lactation or a 48 hr fast, decrease PrRP mRNA expression and reduce the number of cNTS neurons that express detectable PrRP immunoreactivity (Lawrence et al., 2000; Morales and Sawchenko, 2003). These findings suggest that periods of negative energy balance attenuate PrRP expression to thereby reduce the anorexic effect of PrRP signaling (Lawrence et al., 2000; Lawrence et al., 2002) and allow for repletion of energy stores. In light of this, we examined whether a shorter, overnight fast was sufficient to reduce the number of cNTS neurons immunolabeled for PrRP (Chapter 3). However, we observed no effect of fasting or stress treatment on the number of $PrRP^{+}$ neurons (or GLP-1 neurons) (Table 3). These discordant results may be due to the brevity of an overnight fast as compared to the protracted state of negative energy balance produced by lactation or a 48-hour fast.

5.3 ESTABLISHING A CAUSAL ROLE FOR BRAINSTEM NEURONS IN FASTING-MEDIATED ATTENUATION OF STRESS RESPONSES

Substantial evidence demonstrates that NA/GLP-1 receptor antagonism or selective lesion of NA (and likely $PrRP^+$) hindbrain neurons attenuates anxiety-like behavior (Cecchi et al., 2002; Kinzig et al., 2003), stress-induced HPA axis activation (Kinzig et al., 2003; Schiltz and Sawchenko, 2007; Bienkowski and Rinaman, 2008), and stress-induced hypophagia (Rinaman, 1999a; Grill et al., 2004) (Chapter 3)[2]. These studies provide strong evidence that loss of signaling from cNTS neurons may result in attenuated neuroendocrine and behavioral stress responses during fasting. It will be difficult, however, to substantiate the claim that reduced signaling from GLP-1 or $PrRP^+$ A2 neurons *causes* changes in these stress responses during overnight fasting, as this requires physiologically relevant restoration of neurotransmitter signaling in the fasted state. I.c.v or local parenchymal administration of receptor agonists in fasted rats provides a possible experimental route by which to address this hypothesis. However, a number of inherent limitations detract from the utility of this approach:

1. Although agonist administration provides strong foundational evidence of involvement of central receptor signaling in a given process/function, it is exceedingly difficult to both quantify and replicate endogenous neurotransmitter concentrations. Thus, the physiological relevance of agonist administration is questionable and will not provide conclusive causal evidence.

[2] There are currently no available pharmacological antagonists for the PrRP receptor (GPR10), precluding a clear understanding of the role of endogenous PrRP in these processes.

2. As NE and PrRP are expressed in numerous central nuclei, many of which innervate overlapping regions, agonist administration prevents an understanding of the cell groups contributing to any effects observed.
3. Co-localization of vGlut2 in both A2 (Stornetta et al., 2002) and GLP-1 neurons (Zheng & Rinaman, submitted) indicates that these neurons are also glutamatergic, and the use of pharmacological agonists would not recapitulate the dual transmitter release that likely occurs following their activation.

Considering this, additional strategies to replicate endogenous signaling from cNTS GLP-1 and $PrRP^{+}$ A2 neurons are worth considering. The use of optogenetics or designer receptors exclusively activated by designer drugs (DREADD) technology each provide the ability to selectively activate neurons in the fasted state, and it may be possible to adjust/titrate activation to generate physiologically relevant neural output.

Optogenetics involves ectopic expression of light-sensitive ion channels within the CNS, enabling depolarization or hyperpolarization of neural populations with high temporal resolution (Fenno et al., 2011). Using an optogenetic approach in rats, it is possible to inject a lentivirus or adeno-associated virus that expresses channelrhodopsin-2 (ChR2) – a nonspecific cation channel – into the cNTS. By exposing transfected neurons to a blue light (via an implanted optic fiber), it would be possible to drive cNTS neural depolarizations in a fasted rat and observe the consequences of selective "restoration" of signaling from this region on HPA axis activation, anxiety-like behavior, and stress-induced hypophagia (Zalocusky and Deisseroth, 2013). Furthermore, by utilizing opsins engineered to promote membrane trafficking to axon terminals, light activation at specific terminal targets of cNTS neurons may be achieved (e.g., mpPVN,

vlBST, CeA, etc.). This provides a powerful technique by which to uncover the impact of cNTS neural input within specific target nuclei.

A major current limitation to the use of optogenetics in rats is the challenge associated with viral transfection of opsins under the promotion of cell-subtype specific promoters, as these genetic regions are generally too large to package into viral genomes (Zalocusky and Deisseroth, 2013). This limitation currently prevents viral targeting of ChR2 selectively within GLP-1 or $PrRP^{+}$ A2 neurons, and significantly limits the usefulness of this approach. However, the development of a transgenic rat line that expresses the protein Cre recombinase under the control of the endogenous TH promoter (TH:Cre rats) may circumvent this limitation (Witten et al., 2011). In these animals, Cre is expressed in all catecholaminergic neurons, including the A2 neurons of the cNTS (presumably, this has not yet been demonstrated empirically). By using TH:Cre rats, it is possible to generate specific ChR2 expression in A2 neurons through delivery of viruses in which opsin expression is dependent on the cellular presence of Cre (Fenno et al., 2011). Thus, viral injections into the cNTS would allow for selective ChR2 expression within A2 neurons and subsequent, temporally controlled stimulation of these neurons in a fasted rat.

DREADD technology provides another route by which to selectively activate cNTS neurons in fasted rats. DREADD involves the use of a mutated muscarinic receptor that can only be activated by an otherwise pharmacologically inert synthetic ligand (Armbruster et al., 2007; Ferguson et al., 2011). Thus, by coupling the DREADD receptor with a stimulatory G_q protein and virally targeting expression of the receptor to neurons of the cNTS, this technique would allow for neural depolarization following administration of the synthetic ligand (Armbruster et al., 2007; Alexander et al., 2009). A major benefit of DREADD – as opposed to optogenetics – is the ability to systemically administer the synthetic excitatory ligand, obviating the need for

chronic implantation of an optic fiber and thus increasing the tractability of many behavioral and neurophysiological experiments. Use of DREADD, however, is also subject to the challenges associated with viral transfection of the receptor. That is, viral packaging can preclude the incorporation of many larger cell-subtype specific promoters. Thus, the DREADD technique does not allow for specific stimulation of hindbrain GLP-1 or $PrRP^{+}$ A2 neurons at present. While both optogenetic and DREADD stimulation have the potential to clarify the role of GLP-1 or $PrRP^{+}$ A2 neural signaling in fasted rats, technological limitations limit the efficacy of these approaches at present.

Considering the limitations associated with these currently available techniques, it will be challenging to establish the causal role of hindbrain GLP-1 neurons and $PrRP^{+}$ A2 neurons in fasting-mediated changes in central stress responses. Each technology, however, is evolving rapidly and it is possible that development of novel transgenic rat lines will allow for GLP-1- or PrRP-specific neural stimulation through the use of Cre-dependent opsin or DREADD expression in the future. Thus, immediate future directions of this research should be aimed at developing a comprehensive and thorough understanding of 1) the role of brainstem GLP-1 and $PrRP^{+}$ A2 neurons in central stress responses, and 2) the ability of metabolic state to alter the function of these neurons.

5.4 CONCLUSIONS AND FUTURE DIRECTIONS

The research contributing to this dissertation has identified hindbrain GLP-1 neurons and $PrRP^{+}$ A2 neurons as a node for the metabolic tuning of centrally mediated stress responses. However, our current understanding of the interaction between metabolic state and stress responsiveness is

far from complete. While much work remains to be done, a number of immediate future directions will significantly extend current knowledge. First, it is imperative to better understand the functional role of brainstem neurons in generating central stress responses, which can be achieved through numerous lines of experimentation:

1. In Chapter 3, we discovered that antagonism of central GLP-1 signaling prevents cognitive stress-induced hypophagia (Figure 12). Based on previous neuroanatomical and functional work, we hypothesize that this effect is mediated by neurons in the brainstem (Figure 1). By utilizing 4th ventricular administration of GLP-1 receptor antagonist, it can be determined whether cognitive stress-induced hypophagia results from GLP-1 signaling within the hindbrain.
2. With the addition of results presented in Chapter 3, central GLP-1 receptor antagonist has been demonstrated to attenuate anxiety-like behavior, HPA axis activation, and stress-induced hypophagia. Furthermore, GLP-1 signaling within the CeA and mpPVN has been shown to elicit anxiety-like behavior and HPA axis activation, respectively. Despite axonal input to the vlBST, it is unknown whether GLP-1 receptor signaling within this region contributes to anxiety-like behavior or HPA axis activation, and this should be tested directly using local stereotaxic injections of GLP-1 receptor agonists or antagonists.
3. The lack of pharmacological antagonists for PrRP receptors has prevented a clear understanding of the role of endogenous PrRP signaling in central stress responses. One approach to address this dearth of knowledge is the use of RNA interference technology to disrupt either PrRP mRNA expression in A2 neurons or expression of its receptor

(GPR10) within specific brain sites (e.g., mpPVN and vlBST). The use of RNA interference to decrease cNTS PPG mRNA expression has recently been demonstrated (Barrera et al., 2011), providing a platform by which to guide development of this technique.

Second, results from Chapter 4 indicate that ghrelin receptor antagonism is sufficient to partially rescue visceral stress-induced activation of hindbrain GLP-1 neurons and $PrRP^{+}$ A2 neurons in fasted rats. Considering this, it will be important to:

1. Identify and characterize other contributing factors to fasting-mediated reductions in brainstem neural activation, including gastric distention and CORT signaling (Section 5.2.3).
2. Assess the ability of GRA administration to rescue activation of GLP-1 and $PrRP^{+}$ A2 neurons in response to cognitive stress exposure in fasted rats.
3. Determine whether GRA administration reverses fasting-mediated reductions in mpPVN/vlBST neural activation or anxiety-like behavior, two effects predicted by our working model.

Lastly, a more thorough and comprehensive characterization of the metabolic tuning of hindbrain GLP-1 and $PrRP^{+}$ A2 neural stress responses is warranted, including:

1. Examination of the metabolic sensitivity of neural activation in response to additional stressors, including those with differing stimulus intensity, modality, and duration.

2. Clarification of the role of the vagus nerve in metabolic tuning. Our working hypothesis posits that metabolic tuning occurs, at least in part, via the vagus nerve. It might be possible to empirically test this using subdiaphragmatic vagotomy (however, this could only be used to assess metabolic tuning of cognitive stress exposure, as visceral stressors require vagal integrity to elicit central responses)[3].
3. Assessment of metabolic tuning of cNTS neurons by different types of changes in metabolic state, such as chronic food restriction, changes in diet composition, and diet-induced or genetic obesity.

Periods of caloric deficit alter neural, neuroendocrine, and behavioral responses to stress, and it is believed that these alterations represent an adaptive shift in stress responses during periods of negative energy balance. The results of my dissertation work support the hypothesis that metabolic tuning of hindbrain GLP-1 and $PrRP^{+}$ A2 neurons is a mechanism through which negative energy balance decreases central drive to the HPA axis, promotes anxiolysis, and attenuates hypophagic responses to acute stress. This current working model is likely far from complete, and numerous lines of future investigation are essential to improve our understanding of the neural mechanisms responsible for metabolic tuning of central stress responses.

[3] A major caveat of this approach, however, is that vagotomy would also remove a substantial amount of non-metabolic sensory feedback that may shape centrally mediated stress responses. These studies would therefore require carefully designed control groups and conservative interpretation of results.

APPENDIX A

SYSTEMIC LEPTIN DOSE-DEPENDENTLY INCREASES STAT3 PHOSPHORYLATION WITHIN HYPOTHALAMIC AND HINDBRAIN NUCLEI[4]

A.1 INTRODUCTION

Leptin, the peptide product of the *obese* (*Ob*) gene, is released from adipose tissue and the gastric epithelium to signal long- and short-term caloric surfeit, respectively (Zhang et al., 1994; Bado et al., 1998). In this capacity, leptin acts as a hormonal feedback signal to promote negative energy balance (Friedman and Halaas, 1998), in part by increasing sensitivity to satiation signals (Emond et al., 1999; Schwartz and Moran, 2002; Peters et al., 2004; Peters et al., 2005; Peters et al., 2006a) to decrease meal size (Flynn et al., 1998; Kahler et al., 1998; Flynn and Plata-Salaman, 1999; Morton et al., 2005; Kanoski et al., 2012). The strong influence of leptin signaling on energy balance as well as reproductive, autonomic, and other physiological systems has been clearly demonstrated in rodents with recessive mutations in the gene encoding leptin

[4] The entirety of the work presented in Appendix A is from "Systemic leptin dose-dependently increases STAT3 phosphorylation within hypothalamic and hindbrain nuclei," by J.W. Maniscalco and L. Rinaman, 2014, *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 306*, p. R576-R585. Copyright (2014) by the American Physiological Society. Reprinted with permission.

[*ob/ob* mice (Ingalls et al., 1950)] or its receptor [*db/db* mice (Hummel et al., 1966) & *fa/fa* rats (Chua et al., 1996; Iida et al., 1996; Phillips et al., 1996)]. These rodent strains display marked obesity, hyperphagia, autonomic nervous system dysfunction, infertility, and disruption of hypothalamic-pituitary hormonal signaling (Ingalls et al., 1950; Zucker LM, 1961; Zucker and Zucker, 1963; Hummel et al., 1966; Giachetti, 1978; Young and Landsberg, 1983; Chehab et al., 1996; Durham and Truett, 2006).

Leptin exerts its behavioral, metabolic, and hormonal effects, at least in part, via direct action within the central nervous system (CNS). Following peripheral release, leptin is unidirectionally transported into the CNS (Banks et al., 1996) where it can directly access leptin receptors expressed by cells within numerous CNS regions. Leptin receptors are abundantly expressed within the arcuate nucleus of the hypothalamus (ARC), the ventromedial nucleus of the hypothalamus (VMH), and the nucleus of the solitary tract (NTS) (Schwartz et al., 1996; Elmquist et al., 1998a; Grill et al., 2002; Scott et al., 2009), three regions that play integral roles in food intake, body weight regulation, glucose homeostasis, and energy metabolism (Minokoshi et al., 1999; Schwartz et al., 2000; Sternson et al., 2005; Dhillon et al., 2006; Rinaman, 2011; Grill and Hayes, 2012; Maniscalco et al., 2013). Indeed, acute central administration of leptin is sufficient to robustly decrease food intake and body weight (Campfield et al., 1995; Schwartz et al., 1996; Grill et al., 2002), facilitate glucose homeostasis (Minokoshi et al., 1999), and increase sympathetic nerve activity (Dunbar et al., 1997; Casto et al., 1998). Furthermore, central leptin signaling is necessary for normal regulation of body weight and adiposity, since neuronal deletion of leptin receptors results in obesity (Cohen et al., 2001), and CNS-specific rescue of leptin receptors in *db/db* mice ameliorates obesity (Kowalski et al., 2001).

Histological identification of leptin-responsive cells in the CNS can provide a wealth of information regarding the specific cellular phenotypes and neural circuits through which leptin signaling modulates physiology and behavior. The long form of the leptin receptor is a member of the cytokine receptor family, and is coupled to the janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway (Tartaglia, 1997). Leptin binding to this receptor results in phosphorylation of signal transducer and activator of transcription 3 (pSTAT3) (Vaisse et al., 1996), making pSTAT3-immunoreactivity (ir) a useful proxy for identifying cells directly responsive to leptin. Centrally administered leptin increases pSTAT3 immunolabeling within the ARC, VMH, and NTS (Hubschle et al., 2001). However, given that endogenous leptin is secreted from peripheral tissues, the physiological relevance of centrally administered leptin is unclear.

A robust literature indicates that – similar to central leptin administration – systemic administration of leptin has pronounced effects on food intake, body weight, glucose homeostasis, reproductive function, and sympathetic activity (Barash et al., 1996; Dryden et al., 1999; Niimi et al., 1999; Lin et al., 2001; Wetzler et al., 2004). While most studies have used large intraperitoneal or intravenous doses of leptin (i.e., >1 mg/kg BW) to observe these effects, systemic leptin doses ranging from 10-130 μg/kg BW are sufficient to decrease food intake (Campfield et al., 1995; Barrachina et al., 1997; de Lartigue et al., 2010), inhibit gastric emptying (Cakir et al., 2007), and increase sympathetic nervous system (SNS) activity and lipolysis (Shen et al., 2007). Western blot and gel-shift assays indicate that peripheral doses of leptin as low as 50-100 μg/kg BW are sufficient to increase STAT3 phosphorylation centrally (Vaisse et al., 1996; El-Haschimi et al., 2000); however, these techniques do not permit identification of leptin-sensitive cellular populations or circuits. Studies that have identified central pSTAT3-positive cells following systemic leptin administration have used doses ranging

from 1-15 mg/kg BW (Hosoi et al., 2002; Munzberg et al., 2003; Levin et al., 2004; Ellacott et al., 2006; Huo et al., 2007; Huo et al., 2008; Roth et al., 2008; Becskei et al., 2010; Turek et al., 2010; Bouret et al., 2012; Ha et al., 2013), much higher than doses required for behavioral, metabolic, and hormonal responses. Central pSTAT3 immunolabeling following systemic leptin doses below 1 mg/kg BW has not been reported. Considering this, we utilized immunohistochemical antigen retrieval techniques (Munzberg et al., 2003; Levin et al., 2004; Ellacott et al., 2006; Becskei et al., 2010) to reveal pSTAT3-ir within anatomically- and phenotypically-identified populations of CNS cells in rats after peripheral administration of leptin at doses ranging from 50-800 μg/kg BW.

A.2 MATERIALS & METHODS

Animals

Adult male Sprague-Dawley rats (Harlan, IN; 225-275g BW; n = 34) were housed singly in hanging stainless steel wire mesh cages in a temperature-controlled room (20-22°C) on a 12/12 hr light/dark cycle (lights on at 0700 hr). Rats had *ad libitum* access to pelleted chow (Purina 5001) and water, except as noted. All 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.

Injections & Perfusions

Rats were weighed one day before leptin administration to determine proper dosage. Rats were deprived of food (but not water) for 16-18 hours overnight before leptin or vehicle treatment,

since food deprivation increases central responsiveness to systemically administered leptin in mice (Becskei et al., 2010). On the day of the experiment rats were removed from their home cage between 0830 and 1030 hr and injected intraperitoneally (i.p.) with 1.0 ml of sterile 0.15M NaCl containing recombinant rat leptin (Sigma-Aldrich; L5037) as follows: 0 μg/kg BW (n = 4); 50 μg/kg BW (n = 3); 100 μg/kg BW (n = 8); 200 μg/kg BW (n = 6); 400 μg/kg BW (n = 7); 800 μg/kg BW (n = 6). Leptin was dissolved in vehicle just before injection, and rats were returned to their home cage immediately after injection.

Ninety minutes after i.p. injection, rats were deeply anesthetized with pentobarbital sodium (39 mg/1.0 ml i.p., Fatal Plus Solution; Butler Schein) and perfused transcardially with a brief saline rinse followed by fixative (100 ml of 2% paraformaldehyde and 1.5% acrolein in 0.1M phosphate buffer, followed by 100 ml of 2% paraformaldehyde alone)(McLean and Nakane, 1974). Brains were post-fixed *in situ* overnight at 4°C, then removed from the skull and cryoprotected for 24-48 hr in 20% sucrose. Brains were blocked and sectioned coronally (35μm) using a Leica freezing-stage sliding microtome. Sections were collected in six serial sets, and stored at -20°C in cryopreservant solution (Watson et al., 1986) until immunohistochemical processing.

Immunohistochemistry

pSTAT3 Immunolabeling

Immunohistochemical identification of pSTAT3 was used to measure direct leptin signaling in the brain, following a protocol adapted from those described previously (Munzberg et al., 2003; Huo et al., 2004; Levin et al., 2004; Ellacott et al., 2006; Becskei et al., 2010; Bouret et al.,

2012). Free-floating tissue sections were removed from cryoprotectant storage and rinsed thoroughly in 0.02M phosphate buffer (PB). Importantly, our pilot studies indicate that this buffer molarity is essential for optimal pSTAT3-ir. Following rinses, tissue was treated with 0.5% sodium borohydride (20 minutes), 0.3% NaOH + 0.3% H_2O_2 (20 minutes), 0.3% glycine (10 minutes), and 0.03% SDS (10 minutes; all in 0.02M PB). Nonspecific binding was prevented with a 20-minute incubation in blocking solution (0.02M PB containing 4% normal donkey serum, 0.4% Triton-X 100, and 1% BSA) before antibody incubation.

Primary and secondary antisera were diluted in blocking solution. Tissue sections were incubated in rabbit anti-pSTAT3 (1:1,000; Cell Signaling, D3A7). The specificity and selectivity of this commercially available antibody has been reported (Tripathi and McTigue, 2008; Caron et al., 2010). Tissue was incubated in biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch), treated with Elite Vectastain ABC reagents (Vector), and reacted with diaminobenzidine (DAB) intensified with nickel sulfate to produce a blue-black pSTAT3 reaction product.

ARC and NTS Neural Phenotypes

To begin determining the chemical phenotypes of neurons demonstrating pSTAT3-ir following these relatively low doses of leptin, we assessed pSTAT3-ir within ARC neurons expressing the anorexigenic peptide, cocaine- and amphetamine-regulated transcript (CART) (Kristensen et al., 1998). We also assessed pSTAT3-ir within noradrenergic NTS neurons expressing dopamine-β-hydroxylase (DβH) alone or together with prolactin-releasing peptide (PrRP), and in NTS neurons expressing glucagon-like peptide-1 (GLP-1). CART, DβH/PrRP, and GLP1 neurons

were selected for examination because each population has been implicated in central control of body energy balance (Kristensen et al., 1998; Rinaman, 2011; Maniscalco et al., 2013).

To identify pSTAT3-ir within neurons of the ARC that express CART, pSTAT3-labeled forebrain sections from some cases were subsequently incubated in rabbit anti-CART (1:10,000, Phoenix Pharmaceuticals, H-003-62) followed by AlexaFluor 488-conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch) to produce a green fluorescent cytoplasmic signal.

To localize pSTAT3 within DβH- and PrRP-positive neurons of the NTS, pSTAT3-labeled brainstem sections from some cases were incubated in a cocktail of mouse anti-DβH (1:5,000; Millipore, MAB308) and rabbit anti-PrRP (1:1,000; Phoenix Pharmaceuticals, H-008-52). After this, tissue sets were incubated in a cocktail of Cy3-conjugated donkey anti-rabbit IgG (1:300, Jackson ImmunoResearch) and AlexaFluor 488-conjugated donkey anti-mouse IgG (1:300, Jackson ImmunoResearch) to produce red and green fluorescent cytoplasmic signals, respectively.

To localize pSTAT3 within NTS GLP-1-positive neurons, pSTAT3-labeled brainstem sections were incubated in rabbit anti-GLP-1 (1:10,000; Bachem, T-4363) followed by biotinylated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch), Elite Vectastain ABC reagents, and plain DAB to produce a brown cytoplasmic reaction product.

Imaging & Quantification

Quantification of pSTAT3-Expressing Cells in the ARC, dorsomedial (dm)VMH, and NTS

pSTAT3-labeled tissue was visualized using a 20X objective on an Olympus microscope equipped for brightfield and fluorescent optics, and was photographed using a digital camera

(Hamamatsu Photonics, Hamamatsu, Japan). For all anatomical regions, pSTAT3-positive profiles were quantified on images using Adobe Photoshop CS4 image software. The criterion for counting a cell as pSTAT3-positive was the presence of visible blue-black nuclear immunolabeling, regardless of intensity.

pSTAT3-ir was quantified bilaterally at two rostrocaudal levels of the ARC (approximately 2.00mm and 2.45mm caudal to bregma) and bilateral counts were averaged per section. Within the dmVMH, pSTAT3-labeled profiles were quantified bilaterally at a single rostrocaudal level (approximately 2.45mm caudal to bregma). This level represents the core of the nucleus and contained the most robust pSTAT3-ir after our highest peripheral dose of leptin. Within the NTS, pSTAT3-labeled cells were quantified bilaterally at two rostrocaudal levels (approximately 14.36mm and 14.16mm caudal to bregma; see Figure 17) and bilateral counts were averaged per section. In all cases, distance from bregma was approximated based on tissue comparison to a standard rat brain atlas (Swanson, 2004).

Qualitative Assessment of pSTAT3-ir in Phenotypically-Identified ARC and NTS Neurons

pSTAT3-labeled sections co-labeled for CART, DβH/PrRP, or GLP-1 were viewed on the Olympus photomicroscope described above. Using a 20X objective, photographic images were captured from a single selected rostrocaudal level of the ARC (approximately 2.00mm caudal to bregma) or the NTS (approximately 14.36mm caudal to bregma). Neurons were identified using Adobe Photoshop CS4 image software. Criteria for identifying a neuron as CART-, DβH/PrRP-, or GLP-1-positive included clear cytoplasmic labeling and a visible nucleus. Neurons were considered pSTAT3-positive if the nucleus contained pSTAT3 immunolabeling, regardless of intensity.

Statistics

Separate one-way ANOVAs were used to determine the effect of leptin dose (0, 50, 100, 200, 400, and 800 μg/kg BW) on the number of pSTAT3-positive profiles within the ARC, dmVMH, and NTS. When F-values indicated a significant effect, the ANOVA was followed by Fisher's LSD post hoc analyses. Differences were considered significant when $p < 0.05$.

Preparation of Images

Using Adobe Photoshop software, photographic images were adjusted for optimal brightness and contrast. Images that included both immunoperoxidase and immunofluorescence were altered to generate Figure 19B-C (i.e., blue-black NiDAB pSTAT3 labeling photographed in the green color channel was inverted, giving it the appearance of a fluorescent signal). The presence or absence of immunolabeling in images was not digitally manipulated.

A.3 RESULTS

The presence of leptin-induced pSTAT3-ir was assessed throughout the full rostrocaudal extent of the brain, from the upper cervical spinal cord through the prefrontal cortex. Systemically administered leptin elicited robust pSTAT3-ir within the ARC, dmVMH, and NTS. Other brain regions contained only scattered, sparse pSTAT3 labeling, even in rats that received the highest leptin dose.

pSTAT3 Labeling within the ARC

ANOVA indicated a significant effect of all leptin doses on the number of pSTAT3-positive profiles within the ARC [F (5,28) = 35.712, $p < .001$; Figure 15D]. While some pSTAT3-ir was observed in the ARC of vehicle-treated rats, all doses of i.p. leptin significantly increased pSTAT3-ir to the same degree (Figure 15A-C).

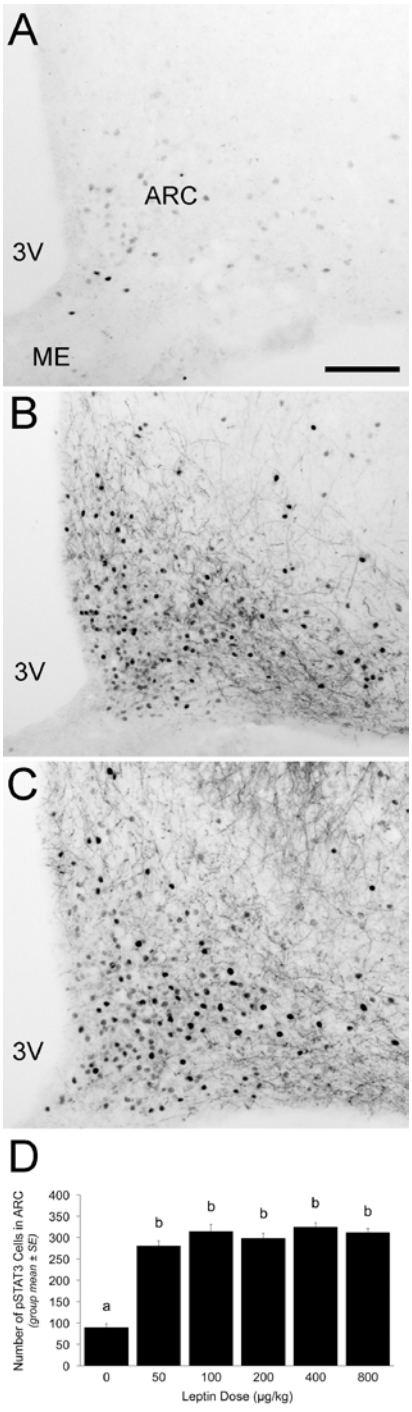

Figure 15. Representative images and summary data of pSTAT3 labeling within the ARC

Representative images of pSTAT3 labeling within the ARC after i.p. injection of (A) saline vehicle, (B) 200 µg/kg BW leptin, or (C) 800 µg/kg BW leptin. Images depict the ARC approximately 2.85 mm caudal to bregma. D, bar

graph illustrating the number of pSTAT3-positive cells within the ARC bilaterally after i.p. leptin at doses from 0-800 μg/kg BW. All doses significantly increased ARC pSTAT3 labeling compared to saline vehicle. Bars with different letters are significantly different ($p < .05$). 3V = third ventricle; ARC, arcuate nucleus of the hypothalamus; ME = median eminence. Scale bar in A = 100 μm, applies also to B and C.

pSTAT3 Labeling within the dmVMH

In contrast to the binary pattern of STAT3 phosphorylation observed in the ARC (i.e., similar increases in pSTAT3-ir after all leptin doses), the dmVMH displayed a leptin dose-dependent increase in pSTAT3 labeling (Figure 16A-C). ANOVA indicated a significant effect of leptin dose on the number of pSTAT3-ir profiles within the dmVMH [F (5,28) = 78.057, $p < .001$]. pSTAT3-ir was negligible in vehicle-treated rats and in rats treated with 50 or 100 μg/kg BW leptin, whereas higher doses elicited marked increases in the number of pSTAT3-ir profiles (Figure 16D).

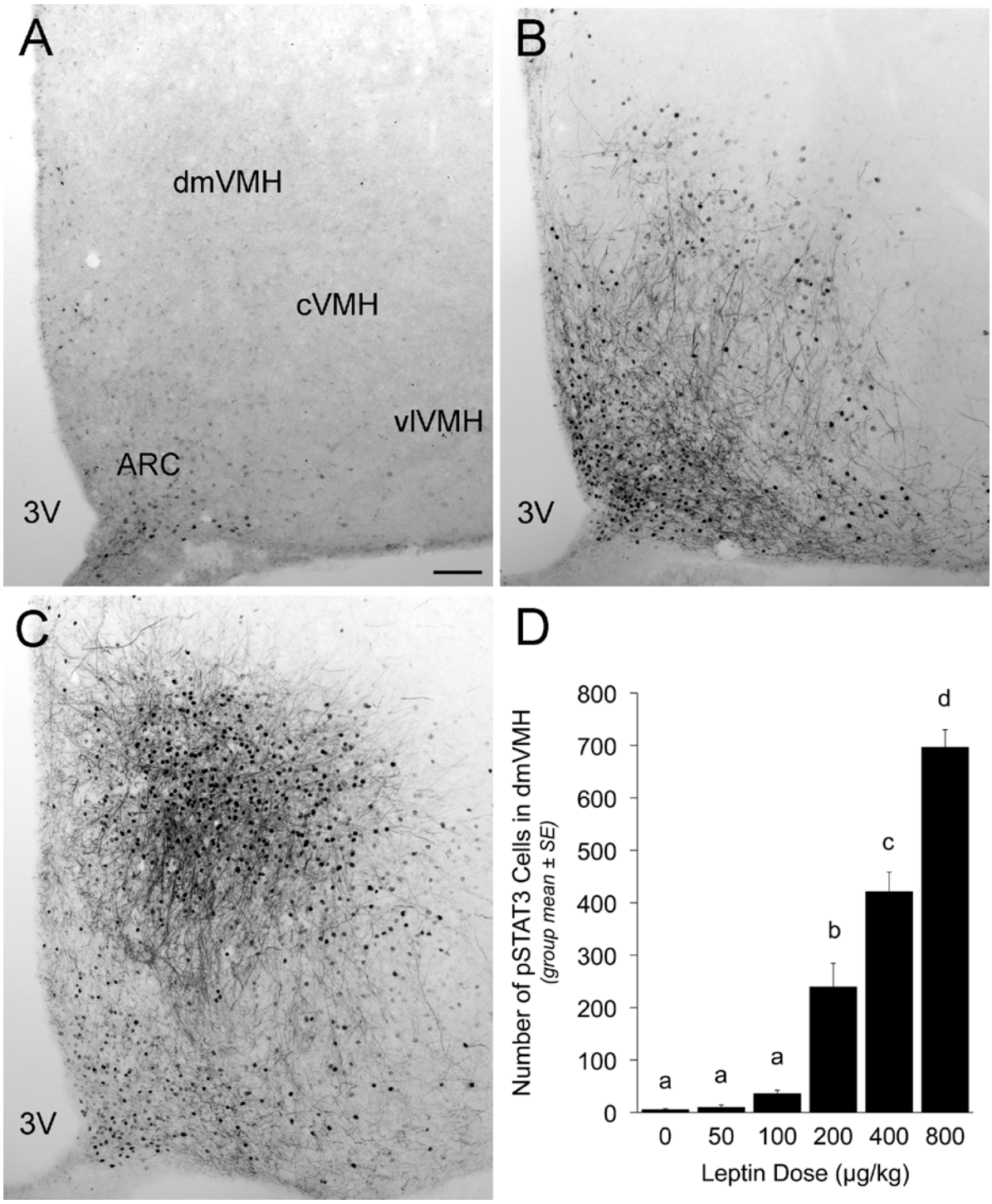

Figure 16. Representative images and summary data of pSTAT3 labeling within the dmVMH

Representative images of pSTAT3 labeling within the dmVMH after i.p. injection of (A) saline vehicle, (B) 200 µg/kg BW leptin, or (C) 800 µg/kg BW leptin. Images depict the dmVMH approximately 2.45 mm caudal to

bregma. D, bar graph illustrating the number of pSTAT3-positive cells within the dmVMH after i.p. leptin at doses from 0-800 μg/kg BW. Leptin dose-dependently increased dmVMH pSTAT3 labeling. Bars with different letters are significantly different ($p < .05$). 3V = third ventricle; ARC, arcuate nucleus of the hypothalamus; dmVMH, dorsomedial subregion of the ventromedial hypothalamus; cVMH, central subregion of the ventromedial hypothalamus; vlVMH, ventrolateral subregion of the ventromedial hypothalamus. Scale bar in A = 100 μm, applies also to B and C.

pSTAT3 Labeling within the NTS

The rostrocaudal extent of the NTS was examined for pSTAT3-ir to evaluate subregional effects of systemic leptin. Even in rats receiving the highest leptin dose (800 μg/kg BW), pSTAT3-ir within the NTS was largely limited to the medial subnucleus of the caudal "visceral" NTS, with peak immunolabeling at the rostrocaudal level of the area postrema (Figure 17). In each rat, STAT3 phosphorylation was quantified in two tissue sections through this level of the NTS (i.e., approximately 14.36 and 14.16mm caudal to bregma) (Figure 17). ANOVA indicated a significant effect of leptin dose on the number of pSTAT3-ir profiles within the NTS at this rostrocaudal level [F (5,28) = 11.282, $p < .001$]. pSTAT3-ir was negligible in vehicle-treated rats, whereas systemic leptin elicited a dose-dependent increase in pSTAT3-ir that reached a plateau at the 200 μg/kg BW dose (Figure 18).

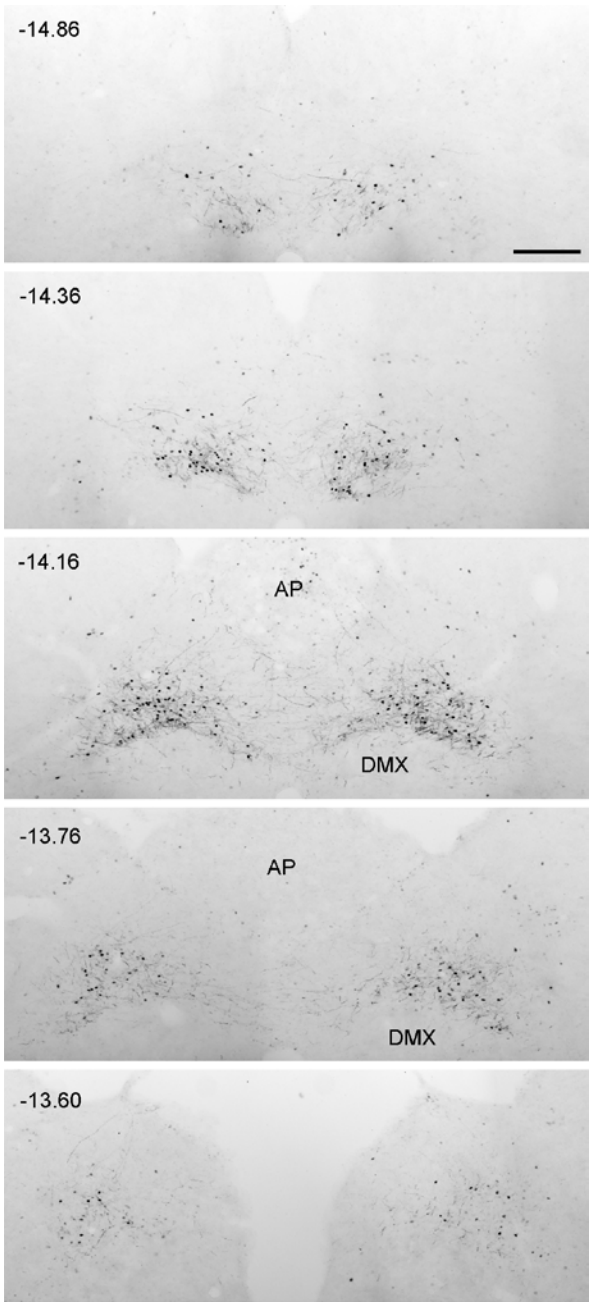

Figure 17. Images of pSTAT3 labeling through the rostrocaudal extent of the caudal NTS after i.p. leptin

Images of pSTAT3 labeling through the rostrocaudal extent of the caudal NTS in a representative rat after i.p. leptin at a dose of 800 μg/kg BW. pSTAT3 immunoreactivity was located primarily within the medial subnucleus of the caudal NTS. Numbers in each panel represent relative distance from bregma. AP = area postrema; DMX = dorsal motor nucleus of the vagus nerve. Scale bar = 200 μm, applies to all panels.

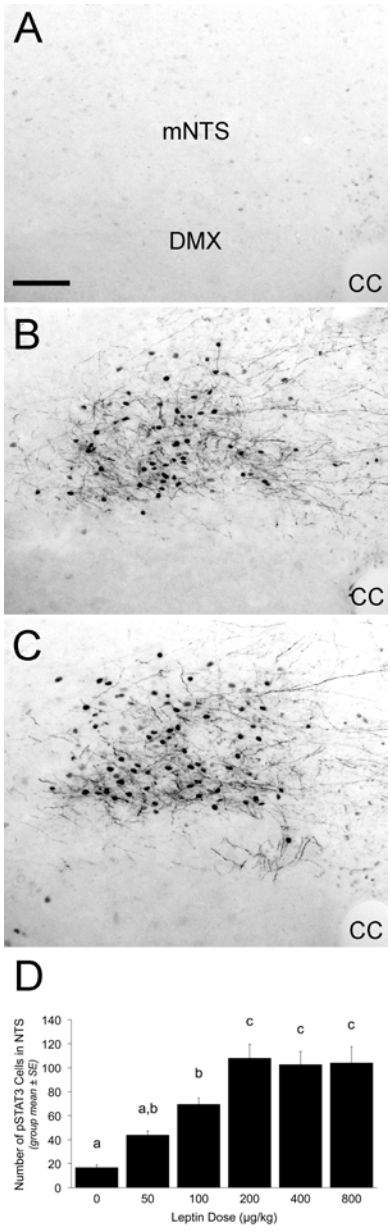

Figure 18. Representative images and summary data for pSTAT3 labeling within the NTS

Representative images and summary data for pSTAT3 labeling within the NTS after i.p. injection of (A) saline vehicle, (B) 200 µg/kg BW leptin, or (C) 800 µg/kg BW leptin. Images depict the caudal NTS approximately 14.36 mm caudal to bregma. D, bar graph illustrating the number of pSTAT3-positive cells within the mNTS after i.p. leptin at doses from 0-800 µg/kg BW. Leptin dose-dependently increased NTS pSTAT3 labeling. Bars with different letters are significantly different ($p < .05$). CC = central canal; mNTS = medial subnucleus of the nucleus of the solitary tract; DMX = dorsal motor nucleus of the vagus. Scale bar in A = 100 µm, applies also to B and C.

pSTAT3 Immunolabeling in Phenotypically Identified Neurons

Consistent with a previous report using peripheral leptin administration in rats (Huo et al., 2008), pSTAT3 within the NTS was not co-localized in any GLP-1 neurons (Figure 19A). pSTAT3-ir was co-localized in a subset of DβH- and DβH/PrRP-positive neurons of the caudal NTS (Figure 19B). However, qualitative assessment indicated that the majority of DβH- and DβH/PrRP-positive neurons did not co-localize pSTAT3, and *vice versa*. Within the ARC, many CART-expressing neurons were also pSTAT3-positive (Figure 19C), consistent with reports that peripheral leptin activates these neurons (Elias et al., 1998; Cone et al., 2001).

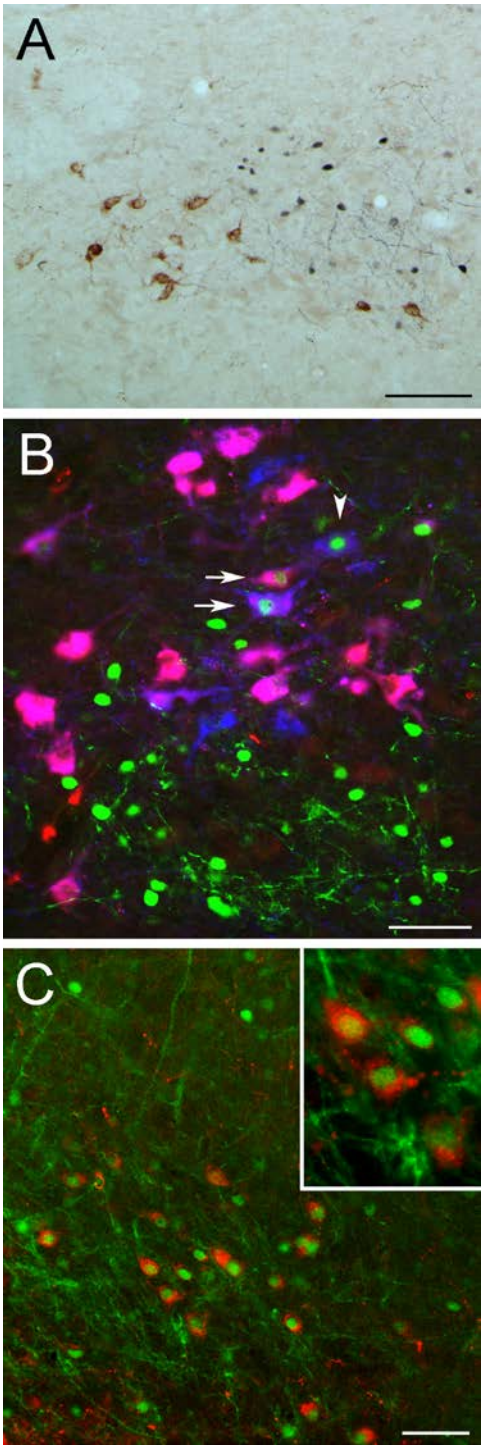

Figure 19. Images of pSTAT3 and phenotypically-identified neurons in the brainstem and hypothalamus

Representative color images of pSTAT3 and phenotypically-identified neurons within the brainstem and hypothalamus. A, pSTAT3 (blue-black) is not colocalized in GLP-1-positive (brown) NTS neurons in rats after any

dose of leptin (this image is from a rat treated with leptin at 200 μg/kg BW; 14.36 mm caudal to bregma). B, pSTAT3 (green) is colocalized in some DβH-positive (blue) and DβH/PrRP-positive (purple-pink) neurons within the caudal NTS after leptin treatment (800 μg/kg BW; approximately 14.36 mm caudal to bregma). The majority of DβH- and DβH/PrRP-positive neurons did not co-localize pSTAT3, and *vice versa*. Arrows indicate pSTAT3+DβH/PrRP-positive neurons; the arrowhead indicates a pSTAT3+DβH-positive neuron that is not immunolabeled for PrRP. C, pSTAT3 (green) is co-localized with many CART-positive ARC neurons (red) after i.p. leptin (400 μg/kg BW; approximately 2.45 mm caudal to bregma). *Inset*, higher magnification view of several double-labeled ARC neurons. Scale bar in A = 100 μm. Scale bars in B and C = 50 μm.

pSTAT3 Immunolabeling within Neural Processes

We observed dose-dependent increases in cytoplasmic pSTAT3-ir within neural processes in the ARC, dmVMH, and NTS. Labeling within the ARC appeared to remain within the anatomic boundaries of the nucleus. Conversely, pSTAT3-positive dmVMH processes projected radially in all directions away from the core of the nucleus, with particularly prominent labeling located ventral to the dmVMH within the internuclear area separating the VMH from the ARC (Figure 16C). Labeling of neural processes within the NTS was most prevalent within the medial subnucleus, but was also observed within the commissural subnucleus and extending into the area postrema (Figure 17 & Figure 18B-C).

A.4 DISCUSSION

Leptin plays a critical role in regulating energy balance and other physiological functions (Moschos et al., 2002; Gautron and Elmquist, 2011; Carlton et al., 2012), and leptin signaling pathways that underlie these effects include direct activation of leptin receptors in brainstem and hypothalamic nuclei (Satoh et al., 1997; Minokoshi et al., 1999; Hayes et al., 2010). In order to better understand how systemically released leptin acts on the brain to modulate physiology and behavior, it is necessary to localize and phenotypically identify leptin-responsive cells within the CNS. The present study is the first to immunohistochemically visualize pSTAT3 in rats after relatively low doses of systemic leptin. Doses used in the present study are known to elicit behavioral and physiological effects after systemic administration (Campfield et al., 1995; Cakir et al., 2007; Shen et al., 2007), and are much lower than systemic doses used previously to induce central pSTAT3 labeling. We report pSTAT3-ir after peripheral doses of leptin as low as

50 μg/kg BW, well within the range of leptin doses that alter physiology and behavior (Campfield et al., 1995; Cakir et al., 2007; Shen et al., 2007). We also conducted the first reported dose-response assessment of leptin-induced pSTAT3 in several brain regions, providing new information regarding leptin sensitivity within and between central nuclei. Our results demonstrate that i.p. leptin at doses ranging from 50-800 μg/kg BW elicit unique and dose-dependent patterns of STAT3 phosphorylation in the ARC, dmVMH, and NTS.

Arcuate Nucleus of the Hypothalamus

It is well established that the ARC plays a critical role in energy balance by transducing circulating signals, including the adiposity signal leptin, into neural responses (Elias et al., 2000; Cone et al., 2001; Cowley et al., 2003). Leptin receptors are abundantly expressed within the ARC, and numerous studies have reported dense ARC pSTAT3-ir in rodents after high peripheral doses of leptin (Levin et al., 2004; Becskei et al., 2010; Turek et al., 2010). However, our study is the first to assess leptin-induced pSTAT3-ir within the ARC after leptin doses lower than 1mg/kg BW. Our results demonstrate that the ARC displays moderate pSTAT3-ir in vehicle-treated rats, presumably the result of endogenous leptin or other signaling factors that phosphorylate STAT under these conditions. Interestingly, the number of pSTAT3-positive ARC cells increased to maximal levels following the lowest leptin dose used in the present study (50 μg/kg BW) – a dose 100-300 times lower than those previously shown to be effective (Hosoi et al., 2002; Levin et al., 2004; Turek et al., 2010). Higher leptin doses elicited no further increase in pSTAT3-ir. These results support the view that the ARC is unusually sensitive to systemically administered leptin, probably due to high rates of peripheral-to-central leptin transport at this site

(Banks et al., 2000b), and likely tied to the ARCs critical role in controlling body energy homeostasis.

Since leptin receptor activation within the ARC decreases food intake and body weight (Satoh et al., 1997), we investigated the ability of peripheral leptin to phosphorylate STAT3 within ARC neurons that have a known anorexigenic role. ARC neurons that express both pro-opiomelanocortin (POMC) and CART (Elias et al., 1998) drive anorexigenic responses to leptin via downstream central targets (Kristensen et al., 1998; Balthasar et al., 2005; Aponte et al., 2011). As predicted, many CART-positive ARC neurons co-localized pSTAT3 after 400 μg/kg BW leptin. This finding is consistent with evidence that POMC/CART neurons contribute to the decreased food intake (Campfield et al., 1995), inhibition of gastric motility (Cakir et al., 2007), and increased sympathetic outflow (Shen et al., 2007) observed following peripheral doses of leptin similar to those used here.

Within the ARC, maximal pSTAT3-ir was observed in rats that received the lowest leptin dose (i.e., 50 μg/kg BW). It will be important in future studies to identify the threshold dose for increased STAT3 phosphorylation within the ARC, and to explore dose-related pSTAT3 responses among phenotypically identified neural populations.

Ventromedial Nucleus of the Hypothalamus

The VMH has long been implicated in the control of feeding and energy balance (Hetherington and Ranson, 1940), and recent evidence indicates that it does so in part through direct leptin signaling. The VMH expresses the leptin receptor (Schwartz et al., 1996; Elmquist et al., 1998a), and intra-VMH leptin injection facilitates blood glucose homeostasis (Minokoshi et al., 1999), increases sympathetic outflow (Satoh et al., 1997), and decreases food intake (Jacob et al., 1997).

Moreover, genetic deletion of the leptin receptor within a subset of VMH neurons that express steroidogenic factor-1 (SF-1) results in increased body weight and susceptibility to diet-induced obesity (Dhillon et al., 2006). The downstream targets through which VMH neurons modulate energy balance remain under investigation (Elmquist et al., 1998b; Sternson et al., 2005).

Following peripheral administration of leptin, dose-dependent increases in pSTAT3-ir were observed within the VMH. pSTAT3-ir was present predominantly within the dorsomedial subregion of the VMH, although labeling was also observed in the central and ventrolateral subregions. Within the dmVMH, doses of leptin at or below 100 μg/kg BW elicited negligible pSTAT3-ir, whereas dose-related increases in STAT3 phosphorylation were observed after administration of leptin at 200, 400, and 800 μg/kg BW. The significant response to leptin at the 200 μg/kg dose is especially noteworthy, as this dose is 25 times lower than the lowest peripheral dose previously reported to increase pSTAT3-ir within the VMH (Turek et al., 2010). No plateau in pSTAT3-ir was observed within the VMH in the present study, suggesting that higher doses of leptin might induce additional STAT3 phosphorylation in this brain region.

The chemical phenotypes of VMH neurons sensitive to leptin at these doses is unclear, although they likely include SF-1 neurons that co-localize pituitary adenylate cyclase-activating polypeptide, based on evidence that these neurons mediate some of leptin's hypophagic effects (Dhillon et al., 2006; Hawke et al., 2009).

Nucleus of the Solitary Tract

A growing literature has implicated neurons of the NTS in the changes in energy balance produced by central leptin signaling [for review, see (Grill, 2010)]. Recent studies show that leptin signaling in the NTS is not only sufficient to decrease food intake and body weight (Grill

et al., 2002) but is necessary for maintenance of normal energy balance in freely-feeding rats (Hayes et al., 2010). Previous reports indicate that systemic leptin doses between 1-5 mg/kg BW are sufficient to elicit STAT3 phosphorylation within the NTS (Munzberg et al., 2003; Ellacott et al., 2006; Huo et al., 2008; Turek et al., 2010). No study, however, had investigated whether low doses of leptin – sufficient to alter autonomic, physiological, and behavioral output (Campfield et al., 1995; Barrachina et al., 1997; Cakir et al., 2007; Shen et al., 2007) – act directly on neurons of the NTS.

The present results demonstrate significantly elevated STAT3 phosphorylation within the NTS in rats after peripheral administration of leptin at doses as low as 100 μg/kg, which is 10-50 times lower than doses used previously. pSTAT3-ir within the NTS displayed a leptin dose-response effect that was intermediate to effects observed within the ARC and the dmVMH. Some baseline pSTAT3-ir was present within the NTS in vehicle-treated rats, and the ability of leptin to increase pSTAT3-ir reached a plateau at the 200 μg/kg BW dose. Increased pSTAT3-ir was confined to the caudal "visceral" NTS, a region known for its role in satiation and visceral sensory processing (Maniscalco et al., 2013). Consistent with a previous report using systemic leptin administration (Huo et al., 2008), most of the pSTAT3-ir observed in the present study was located within the medial subnucleus (mNTS), which receives digestive-related vagal sensory input (Altschuler et al., 1989; Miselis et al., 1991). Intra-mNTS leptin injections suppress food intake and motivation for food seeking (Grill et al., 2002; Kanoski et al., 2013), while virus-mediated knockdown of mNTS leptin receptors increases food intake, meal size, body weight, and adiposity (Hayes et al., 2010; Kanoski et al., 2012). However, the neural circuits underlying these effects remain unclear.

The majority of pSTAT3-ir within the caudal NTS was localized in phenotypically unidentified neurons. We were somewhat surprised to observe relatively few DβH- and DβH/PrRP-positive neurons among those expressing pSTAT3-ir, because these neurons together comprise the A2 noradrenergic cell group, which contributes importantly to food intake and meal size control in rats (Rinaman, 2011; Maniscalco et al., 2013). GLP-1 neurons were never pSTAT3-positive, consistent with a previous report in rats, but in contrast to histological and electrophysiological results in mice (Huo et al., 2008; Hisadome et al., 2010). This is not to say, however, that leptin does not affect A2 or GLP-1-expressing neurons in rats. Leptin exerts a strong depolarizing effect on glutamatergic vagal afferents that synapse directly onto neurons of the NTS (Peters et al., 2004; Peters et al., 2006c; Appleyard et al., 2007), providing a potential route through which leptin receptors might modulate visceral afferent signaling to caudal NTS neurons, including A2 and GLP-1 neurons.

Dose Response Summary

To our knowledge, this report is the first to describe differential dose-related sensitivity of central pSTAT3-ir responses to leptin. The ARC displayed the greatest sensitivity, with maximal pSTAT3 labeling observed after the lowest leptin dose administered (i.e., 50 μg/kg BW), while pSTAT3-ir within the NTS did not reach maximal levels until rats were dosed with leptin at 200 μg/kg BW. Both the ARC and the NTS displayed leptin dose-related plateaus in pSTAT3-ir, evidence that higher doses would be unlikely to further increase pSTAT3-ir in these regions. Phosphorylation of STAT3 within the dmVMH, however, increased progressively through the highest dose administered (i.e., 800 μg/kg BW) without reaching an evident plateau, suggesting that higher doses might produce additional increases in pSTAT3-ir within the VMH.

Cytoplasmic pSTAT3 Immunoreactivity

In addition to nuclear labeling, strong pSTAT3-ir was present within neural processes. Leptin receptors are expressed on both proximal and distal dendrites, where STAT3 phosphorylation occurs before translocation to the nucleus (Banks et al., 2000a; Villanueva and Myers, 2008; Ha et al., 2013). Thus, neuritic labeling likely represents dendritic pSTAT3 that has yet to undergo nuclear translocation. Since pSTAT3 accumulates in the cell's nucleus but not in the cytoplasm, it is likely that visualization of dendritic pSTAT3-ir requires more sensitive immunohistochemical and optic techniques than are commonly used (Ha et al., 2013). Our report is not the first to show dendritic pSTAT3 labeling (Hubschle et al., 2001; Hosoi et al., 2002; Roth et al., 2008; Ha et al., 2013), but immunolabeling in the present study is significantly more pronounced than previously documented.

Importantly, the dendritic pSTAT3 labeling in our material closely matches established dendritic patterns of neurons within each brain region. Golgi staining reveals that ARC neuronal dendrites remain largely within the anatomical boundaries of the ARC (Millhouse, 1979), whereas VMH neuronal dendrites radiate in all directions, including ventrally-directed dendritic branches that extend into the internuclear area separating the ARC from VMH (Millhouse, 1973, 1979). These dendritic patterns precisely match the pSTAT3 labeling we observed within the medial hypothalamus. Within the NTS, dendritic pSTAT3 immunolabeling was confined predominantly to the medial subnucleus, but was also observed in processes extending medially into the commissural subnucleus and dorsally into the area postrema. This pattern of labeling reflects the organization of NTS neuronal dendrites (Ellacott et al., 2006), and is consistent with

dendritic pSTAT3 labeling reported within the NTS of rats that received a much higher systemic dose of leptin (Hosoi et al., 2002; Ha et al., 2013).

Perspectives and Significance

Since its discovery 20 years ago, much has been learned about leptin and leptin receptor-mediated regulation of physiology and behavior. However, much remains to be discovered regarding the structure and function of neural circuits through which leptin elicits its diverse effects. The present study documents STAT3 phosphorylation within the rat CNS after systemic doses of leptin that are up to 300 times lower than those used previously to generate pSTAT3-ir. Furthermore, this study provides the first dose-response assessment of leptin-induced STAT3 phosphorylation within the ARC, VMH, and NTS, providing new information regarding leptin sensitivity within and between these nuclei. Additional studies will be necessary to further characterize the neurochemical phenotypes and axonal projections of central neurons in which STAT3 is phosphorylated by systemic leptin. Further characterization of central leptin-sensitive neurons and circuits will lead to a better understanding of how peripherally-derived leptin acts centrally to modulate energy balance and numerous other physiological functions.

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)[5]

B.1 INTRODUCTION

Factors that increase or decrease food intake do so by altering meal size, meal frequency, or both (Smith, 1998; Smith, 2000, 2004). 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, 1978; Seeley et al., 1994; Grill and Kaplan, 2002; Grill,

[5] The entirety of the work presented in Appendix B is from "Satiation and stress-induced hypophagia: Examining the role of hindbrain neurons expressing prolactin-releasing peptide or glucagon-like peptide 1," by J.W. Maniscalco, A.D. Kreisler, and L. Rinaman, 2013, *Frontiers in Neuroscience, 6:199*.

2010), and in neonatal rats with functionally immature forebrain-brainstem connections (Hall and Bryan, 1980; Hall and Swithers-Mulvey, 1992; 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 (Smith, 1998; Smith, 2000, 2004). 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 (cNST; 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 cNST, and the caudal medullary reticular formation (Larsen et al., 1997b; Merchenthaler et al., 1999)[6]. 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., 1998b; Lawrence et al., 2000; Kinzig et al., 2002; Lawrence et al., 2002; Schick et al., 2003; Grabauskas et al., 2004; Lawrence et al., 2004;

[6] Amoeboid microglia also contain GLP-1 immunoreactive material, at least when activated in culture (Iwai et al., 2006), 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.

Bechtold and Luckman, 2006; Nakade et al., 2006; Takayanagi et al., 2008; Holmes et al., 2009; Takayanagi and Onaka, 2010; Hayes et al., 2011; 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 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.

B.2 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 NST and the nearby medullary reticular formation (Figure 20). The cNST is the “visceral” NST, distinct from the more rostral “gustatory” NST (Lundy-Jr. and Norgren, 2004). The cNST 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, 2003b; 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 cNST 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 cNST (Shapiro and Miselis, 1985a; Kachidian and Pickel, 1993; Cunningham-Jr. et al., 1994), and cNST neurons innervate other NST 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 cNST (Shapiro and Miselis, 1985b).

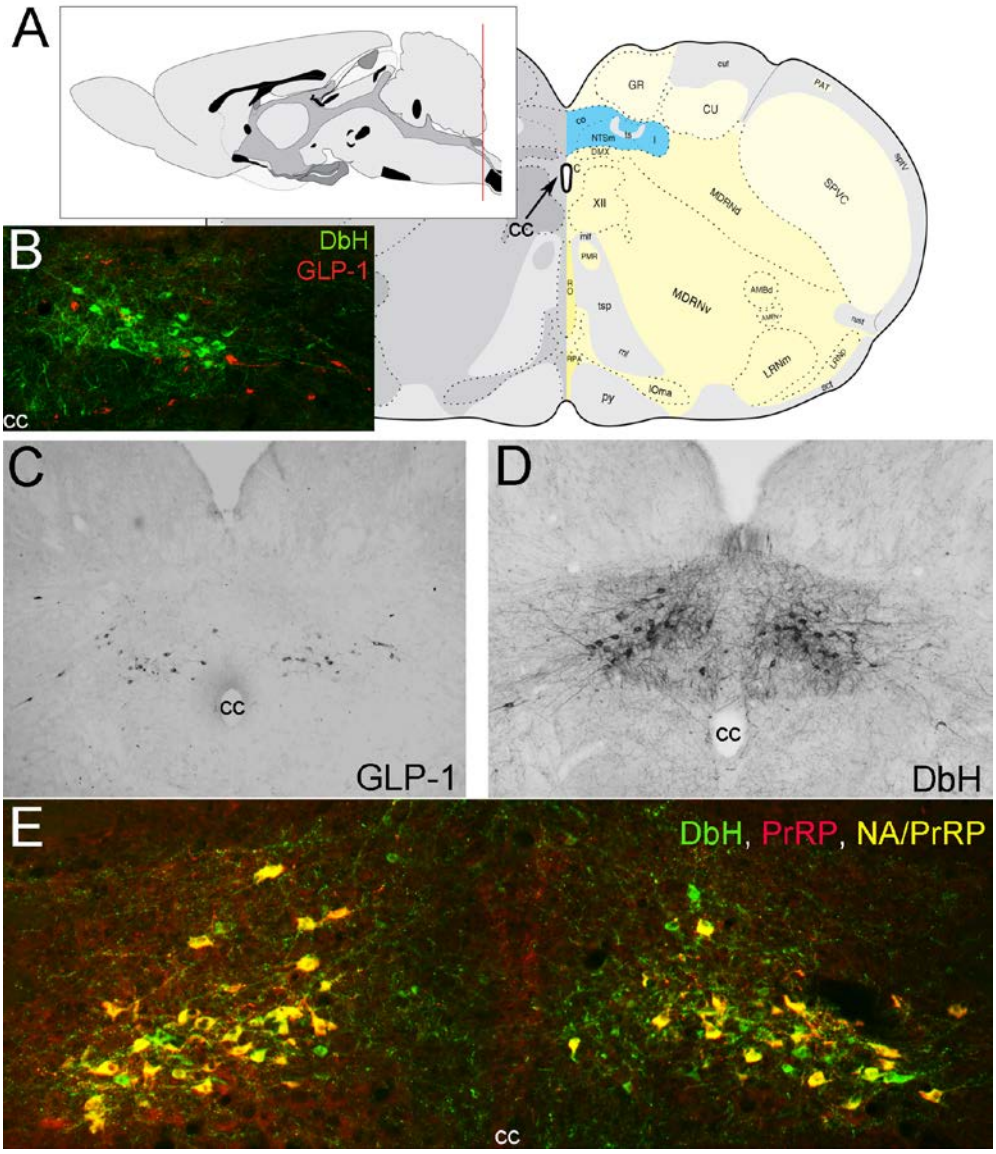

Figure 20. Location of PrRP and GLP-1 neurons in the rat hindbrain

Location of PrRP and GLP-1 neurons in the rat hindbrain. **A,** Schematics illustrating the location of the cNST (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 1 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.*

In addition to inputs from the AP, cNST 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 cNST neurons, including GLP-1 and NA neurons, the latter of which undoubtedly includes the PrRP-positive majority subpopulation (Altschuler et al., 1989; 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 cNST, providing high-fidelity transmission of sensory nerve activity. Other visceral and somatic sensory inputs are relayed to the cNST from the spinal cord, trigeminal and related nuclei, and reticular formation (Menétrey and Basbaum, 1987; Arbab et al., 1988; Altschuler et al., 1989; 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 et al., 1993; Chan and Sawchenko, 1994; Rinaman et al., 1997; Chan and Sawchenko, 1998; Rinaman et al., 1998; Dayas et al., 2001b; 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).

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 cNST 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 and Day, 2001; Taché et al., 2001; Buller et al., 2003; 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; Onaka et al., 1995; Blessing, 1997; Rinaman and Schwartz; Onaka et al., 2010).

Beyond the cNST.

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 cNST, 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 cNST are functionally distinct from those located within the reticular formation. For example, NA and GLP-1 neurons within the cNST receive direct visceral sensory input (Appleyard et al., 2007; Hisadome et al., 2011), whereas those in the medullary reticular formation do not. This may explain why A2 NA neurons within the cNST 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 cNST 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 cNST A2 cell group (Li et al., 2009). There is no published evidence that GLP-1 neurons within the cNST 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 cNST 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 cNST 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 cNST 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.

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 20 for colocalization of PrRP and DbH immunolabeling within the cNST). 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

NST 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 cNST 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 et al., 1988; Sawchenko et al., 1990; Sawchenko and Pfeiffer, 1995). Apparently, none of these signaling molecules are expressed by cNST 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 cNST 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 4^{th} 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 21). Additional support comes from a transneuronal viral tracing study demonstrating that cNST 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 cNST neurons that are synaptically linked to ingestive pattern generators and oral motor output circuits.

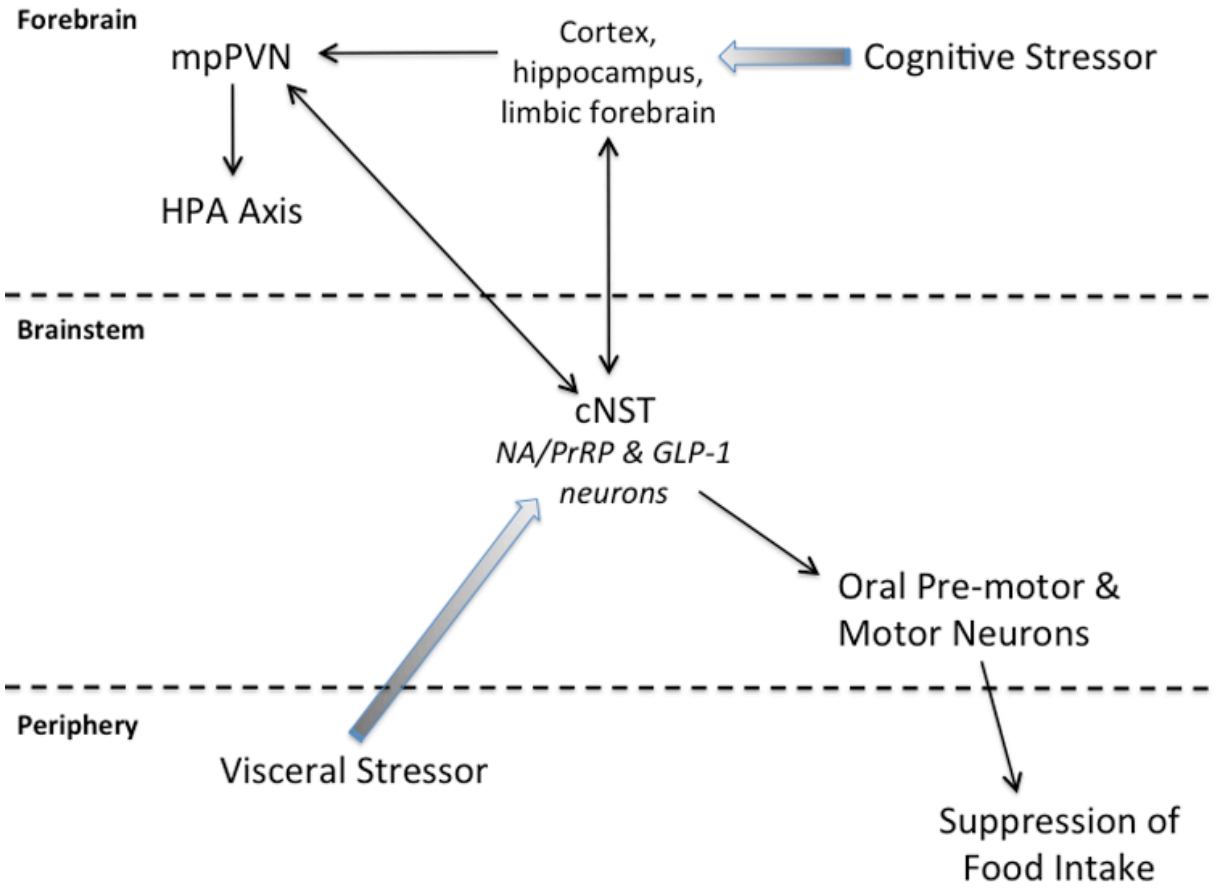

Figure 21. Summary schematic of our working hypothesis

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 cNST. Visceral stressors typically are unconditioned stimuli that first engage cNST neurons (including PrRP and GLP-1 neurons) that innervate hypothalamic and limbic forebrain targets to recruit the HPA axis. Neurons within the cNST engage brainstem targets to organize ingestive motor output.

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 cNST. 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., 1986a; Matsumoto

et al., 2000; Sarkar et al., 2003b) 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, 2003a; Ritter et al., 2003; Rinaman and Dzmura, 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 and Comer, 2000), and perhaps to guard against the overconsumption of unanticipated and/or excessively large meals.

As discussed in the Introduction, 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).[7] 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, 1978; Hall and Bryan, 1980; Hall and Swithers-Mulvey, 1992; Rinaman et al., 1994; Seeley et al., 1994; Grill and Kaplan, 2002). The following section reviews evidence that PrRP and GLP-1 signaling pathways participate in satiation, and the final section reviews evidence that similar signaling pathways contribute to stress-induced hypophagia.

B.3 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 cNST, the former presumably including PrRP-positive neurons, are

[7] HPA axis recruitment by PrRP and GLP-1 signaling pathways is discussed further below.

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, 1999b; 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., 2003; 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 cNST 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 (Takayanagi et al., 2008; Maniscalco et al., 2012). Conversely, in alternate tissue sections from the same satiated rats, approximately 20% of identified GLP-1 neurons express cFos (Maniscalco et al., 2012). 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., 2012). 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 cNST 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, 1999b). 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.

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., 2003), perhaps because intake in deprived rats already is quite high. Conversely, Ex-9 injections targeted to the 4th ventricle or cNST 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 cNST/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).

B.4 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 cNST 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, 1999b, 2003a; 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 cNST 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, 2003a; Rogers et al., 2003; Grabauskas et al., 2004; Rinaman, 2004; Nakade et al., 2006; 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 21). Cognitive stressors originate from conditioned and unconditioned cues that are processed through the cortex and hippocampus before engaging the limbic forebrain and hypothalamus (Figure 21). 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., 2012), likely due to recruitment of descending inputs to the cNST that arise from stress-sensitive regions of the hypothalamus and limbic forebrain (Dayas and Day, 2001; Buller et al., 2003; Dayas et al., 2004; Blevins and Baskin, 2010) (Figure 21). 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 cNST,

or indirectly via descending projections from the hypothalamus and limbic forebrain (Figure 21). 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 cNST (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 cNST neurons (Zittel et al., 1999), including NA neurons (Maniscalco et al., 2012) 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, 2003a). Interestingly, DbH -/- mice (which cannot convert dopamine to NE) 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 cNST PrRP neurons (Rinaman, 2003a). 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, 1999a; 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 cNST (Thiele et al., 1998a), evidence that GLP-1R signaling contributes to LiCl-induced recruitment of cNST 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 and Rivest, 2003), and signaling through both routes engages cNST 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, 1999b). PVN and HPA axis activation in response to immune challenge is significantly attenuated in rats after unilateral transection of ascending projections from the cNST 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., 2001b; Dayas et al., 2001a; Maruyama et al., 2001; Banihashemi et al., 2011), apparently via descending projections from the PVN (Dayas and Day, 2001; Dayas et al., 2004). Recent findings in our laboratory indicate that restraint also activates GLP-1 neurons in rats (Maniscalco et al., 2012). 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.

B.5 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 cNST 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.

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