INVESTIGATING THE ENTEROENDOCRINE – BRAIN AXIS: GHRELIN CELL AND ECL CELL PHYSIOLOGY AND GHRELIN ACTION ON MOOD AND COMPLEX EATING

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DEDICATION

I would first of all like to dedicate this work to my loving parents for their never-ending support and belief that I can accomplish anything. My mother has always provided me a shoulder to lean on when I am struggling, a hand to wipe away my tears when tragedy strikes, and a heart that generously gives more love than I could ever ask for. As I have grown and matured, she has become more than just a mother—she has become my best friend. I am also forever indebted to my father. He has always encouraged me to follow my dreams, never pressuring me with demands, but rather nurturing my strengths with positive reinforcement and tenderness that can only come from a devoted parent. My father served as the perfect model for a hard-working, responsible individual, selflessly working hard to provide our family with every opportunity possible. My parents have instilled in me the motivation, desire, and dedication to excel at every task at hand. I could never have become the person I am today without them. Secondly, I would like to thank David Barry for supporting me throughout my graduate school career. He has been a calming source during stressful times and is the first person to celebrate any of my accomplishments. Sharing his unwavering strength with me, David has made me more resilient when dealing with hardships. He has also shared with me such a compelling passion for science that is has opened my eyes to the beauty inside a whole range of scientific topics. I could not write this dedication without including a deep thanks to my dog, Hugo. Even after enduring lonely nights when I have to spend long hours in the lab, Hugo is always ready to greet me with a wagging tail upon my arrival home. He has been a great source of happiness and joy in my life, and I cannot picture these past years without him. I would also like to recognize Dr. Jeffrey Zigman and Dr. Amelia Eisch. As my mentor, Dr. Zigman has pushed me to exceed all expectations and become a productive, independent scientist. He has allowed me to expand my knowledge on a diverse range of subjects and techniques, thoroughly developing my scientific skills and teaching me how to overcome obstacles. Dr. Eisch has been very helpful as well, serving as my committee chair and advising me throughout different stages of graduate school. I truly appreciate the support I have received from her as well as from the members of her lab.

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Abstract

The mechanisms and neurochemical pathways through which the orexigenic peptide hormone ghrelin act to regulate homeostatic feeding is fairly well documented. However, less understood are the mechanisms and brain regions that mediate ghrelin's effects on mood and complex eating behaviors. At the cellular level, little is known about the ghrelin cell's transcriptional profile, its secretory products other than ghrelin, and its relationship to other gastric endocrine cells, such as the histamine producing enterochromaffin-like cell. My doctoral research encompasses multiple aspects of the ghrelin system, from physiological assessments of the ghrelin cell to evaluations of ghrelin action on cue-potentiated feeding and stress-induced

depressive-like behavior. Ghrelin has antidepressant effects, which become obvious following chronic stress. In the first part of my thesis, I found that this effect was mediated by neurogenesis. I observed that chronic stress reduces neurogenesis more severely in the ventral dentate gyrus of *Ghsr*-null mice, suggesting ghrelin provides a level of neuroprotection in the stress environment. Administration of anti-apoptotic P7C3-related compounds not only blocked stress-induced reductions in neurogenesis, but also minimized the severity of depressive-like behavior in mice. Focal hippocampal irradiation prevented the anti-depressant efficacy of P7C3-related compounds, indicating that P7C3 regulates mood directly through neurogenesis. In the second part of my thesis, I designed a novel protocol for studying cue-potentiated feeding behaviors in mice. Absence of ghrelin signaling in *Ghsr*-null mice, or administration of a ghrelin receptor antagonist in wild-type mice, disrupted the development of normal cue-food associations. Additionally, I discovered *Ghsr* expression in the basolateral amygdala (BLA), and BLA neuronal activation in response to a food-associated positive cue significantly correlated with amount of food intake. Thus, ghrelin signaling in the BLA may be responsible for its mediation of cue-potentiated feeding behaviors. The third part of my thesis examined the ghrelin cell transcriptome for potential secretory proteins and revealed significant expression of *Rbp4*, *Ttr*, and *Nucb2*, along with RBP4 protein secretion. Lastly, I characterized a novel HDC-Cre mouse model that may be advantageous in future studies to determine potential interactions between histaminergic and ghrelin signaling pathways. The full range of these discoveries advances our comprehensive understanding of ghrelin.

TABLE OF CONTENTS

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LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

AC3 – activated caspase 3

Acyl ghrelin – active form of ghrelin, has post-translational octanoylation of serine-3

AgrP – agouti-related peptide

ANOVA – analysis of variance

ARC – arcuate nucleus

Arc – product of immediate early gene, mRNA expression occurs after neuronal activation

BDNF – brain-derived neurotrophic factor

BLA – basolateral amygdala

BrdU – bromodeoxyuridine, thymidine analog

c-Fos – product of immediate early gene, mRNA expression occurs after neuronal activation

CNS – central nervous system

Compound 26 – ghrelin receptor antagonist

CPP- conditioned place preference

CR – caloric restriction

CREB – cyclic AMP response element binding protein, transcription factor

CS+ - positive conditioned stimulus

CS- - negative conditioned stimulus

CSDS – chronic social defeat stress

C_T – comparative threshold cycle value, measurement output in qPCR analysis

DCX – doublecortin, a marker of proliferative neuroblasts and immature neurons

DEPC – diethylpyrocarbonate

DG – dentate gyrus of the hippocampus

DNA – deoxyribonucleic acid

DRD2 – dopamine receptor subtype 2

ECL – enterochromaffin-like

eGFP – enhanced green fluorescent protein

EPM – elevated plus maze, measure of anxiety-like behavior

ERK – extracellular signal-regulated kinase

FACS – fluorescence-activated cell sorting

FST – forced swim test, measure of antidepressant behavior

FTO – fat mass and obesity associated protein

FTO – gene for FTO

GCL – granule cell layer of DG

GH – growth hormone

Ghrelin-hrGFP mice – transgenic mice expressing hrGFP under the control of the ghrelin promotor

Ghrl – gene for ghrelin

GHSR – growth hormone secretagogue receptor, ghrelin receptor

Ghsr – gene for GHSR

Ghsr-null or GHSR-null – mice with a transcriptional blocker preventing *Ghsr* transcription

GIF – gastric intrinsic factor

GLUT4 – glucose transporter 4

GOAT – ghrelin-c-acetyl transferase, enzyme that modifies des-acyl ghrelin to acyl-ghrelin

Goat – gene for GOAT

HDC – histidine decarboxylase, enzyme involved in histamine synthesis

HDC-cre – transgenic mice expressing Cre recombinase under control of the HDC reporter

HDC/Tmt mice – HDC-Cre mice crossed with Tmt reporter mice

HFD – high fat diet

Homer1a – product of immediate early gene, mRNA expression occurs after neuronal activation

HPA – hypothalamic-pituitary-adrenal

hrGFP – humanized *Renilla reniformis* green fluorescent protein

ICV – intracerebroventricular

IHC – immunohistochemistry

IP – intraperitoneal

Ki67 – nuclear protein often serving as a marker for proliferating cells

LH – lateral hypothalamus

LV – lateral ventricle

MAPK – mitogen activated protein kinsase

ML – molecular layer of DG

mPFC – medial prefrontal cortex

mRNA – messenger ribonucleic acid

NAc – nucleus accumbens

NDS – normal donkey serum

NeuN – neuronal nuclei, a marker for mature neurons

NPY – neuropeptide Y

NUCB2 – nucleobindin 2, precursor for nesfatin-1

Nucb2 – gene for NUCB2

OB – olfactory bulb

OF – open field test

P7C3 – aminopropyl carbazole with neuroprotective properties

P7C3-A20 – analog of P7C3 with stronger neuroprotective effects

P7C3-S184 – analog of P7C3 lacking proneurogenic properties

PACAP - pituitary adenylate cyclase activating peptide

PAC1 – PACAP type 1 receptors

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

pCREB – phosphorylated CREB

pERK – phosphorylated ERK

PG-1 – cell line obtained from pancreatic ghrelinomas

PFA – paraformaldehyde

PFC – prefrontal cortex

Prox1 – expressed highly in DG, marker of intermediate progenitor cells and young granule cells

qRT-PCR – quantitative real-time polymerase chain reaction

RBP4 – retinol binding protein 4

Rbp4 – gene for RBP4

RESP18 – regulated endocrine specific protein 18

RMS – rostral migratory stream

RNA – ribonucleic acid

RT – room temperature

SB334867 – orexin receptor 1 selective antagonist

SDS – sodium dodecyl sulfate

SEM – standard error of mean

SG-1 – cell line obtained from stomach ghrelinomas

SGZ – subgranular zone of dentate gyrus

SVZ – subventricular zone

TH – tyrosine hydroxylase

Tmt – tomato fluorescent protein

Tpsb2 – tryptase beta 2

TSA – tyramide signal amplification reagants

TTR – transthyretin protein

Ttr – gene for transthyretin

VIP – vasoactive intestinal peptide

VTA – ventral tegmental area

WAT – white adipose tissue

WT – wild-type

CHAPTER ONE

Introduction

Since the discovery of the orexigenic hormone peptide ghrelin, 15 years of experimentation have led to thousands of ghrelin-related publications. After the very first studies conveying ghrelin's crucial role in growth hormone (GH) secretion, appetite, and adiposity, researchers elaborated on the magnitude of its importance to cover an expanse of biological roles outside of homeostatic feeding behavior. Currently, ghrelin has been implicated in affecting mood regulation, reward-related behaviors, learning and memory, cardioprotection, gastrointestinal function, inflammatory responses, glucose and energy homeostasis, bone metabolism, and muscle atrophy (Andrews, Z.B. 2011; Perello, M. et al. 2012; Sakata, I. et al. 2012; Sato, T. et al. 2012; Scott, M.M. et al. 2012; Peeters, T.L. 2013; Pradhan, G. et al. 2013; Uchida, A. et al. 2013). My collection of experiments embraces all aspects of ghrelin, whether examining ghrelin action on mood and habitual eating from a systemic viewpoint or probing the physiology of the ghrelin cell for a more local perspective. The main focus of my thesis work involves aspects of ghrelin signaling in psychiatric mood disorders as well as hedonic feeding behaviors such as habitual eating. Additionally, my studies include the assessment of potential new secretory peptides in the ghrelin cell and the characterization of the histaminergic system in a new mouse model, which allows for the easier study of interactions between ghrelin cells and histaminergic enterochromaffin-like (ECL) cells within the stomach.

The discovery of ghrelin and its relationship to other enteroendocrine cell types

The events from 1996-1999 that led to the initial discovery of the hormone peptide ghrelin through reverse pharmacology quickly led to an abundance of exciting new studies within the first year of its discovery (Howard, A.D. et al. 1996; Kojima, M. et al. 1999; Date, Y. et

al. 2000; Date, Y. et al. 2000; Dieguez, C. et al. 2000; Peino, R. et al. 2000; Seoane, L.M. et al. 2000). It began with the cloning of a novel G-protein coupled receptor upstream of GH release, located in the anterior pituitary (Howard, A.D. et al. 1996). As a side note, it was already well established in the field that GH secretion from the pituitary promotes overall cell growth, reproduction, and regeneration in many different types of tissues such as muscle, bone, and internal organs (Simpson, M.E. et al. 1947; Evans, H.M. et al. 1948; Evans, H.M. et al. 1948; de, J.S. et al. 1950). GH had also been shown to stimulate lipolysis and gluconeogenesis, so upstream mechanisms for GH regulation were of particular interest (Fain, J.N. et al. 1971; Rogers, S.A. et al. 1989; Emmison, N. et al. 1991). This novel receptor upstream of GH secretion, designated the growth hormone secretagogue receptor type 1a (GHSR-1a, or GHSR), was a 7 transmembrane protein coupled to the Gq subfamily of heterotrimeric GTP-binding proteins (Howard, A.D. et al. 1996). Three years after cloning the first GHSR, a 28 amino acid hormone peptide, ghrelin, was discovered as its endogenous ligand (Kojima, M. et al. 1999). Notably, a truncated form of the GHSR, known as GHSR-1b, is not activated by ghrelin and has no known ligand, but has been implicated in modifying GHSR-1a activity through heterodimer formation (Chow, K.B. et al. 2012).

Secreted from X/A-like endocrine cells in the rodent gastrointestinal (GI) tract, ghrelin undergoes a unique post-translational modification of *O*-*n*-octanoylation at serine 3, resulting in its bioactive form, acyl ghrelin (Date, Y. et al. 2000; Hosoda, H. et al. 2000; Hosoda, H. et al. 2000; Yang, J. et al. 2008). Notably, these X/A-like gastric cells, known as P/D_1 cells in humans, had previously been distinguished due to their distinct morphological features and electron-dense secretory granules (Grube, D. et al. 1979; Simonsson, M. et al. 1988; Date, Y. et al. 2000; Rindi, G. et al. 2002; Rindi, G. et al. 2004; Yabuki, A. et al. 2004; Mizutani, M. et al. 2009). Initially observed in the rat, these X/A-like cells were named “X” due to their unknown function at the time of discovery, and “A-like” because of their resemblance to pancreatic A-cells

(Rindi, G. et al. 2004). Although X/A-like cells are also localized to the mucosal layer of the duodenum, ileum, cecum, and colon, an overwhelming majority are found in the gastric body of the stomach (Sakata, I. et al. 2002). The morphological features of these cells reveal round to ovoid cells with round, compact, electron-dense granules (Grube, D. et al. 1979; Rindi, G. et al. 2002). Additionally, X/A-like cells exist as both closed-type cells without luminal contact (in the stomach) and open-type cells with their apical cytoplasmic processes contacting the lumen (in the other regions of the GI tract) (Sakata, I. et al. 2002; Sakata, I. et al. 2010). This suggests that there may be different signaling mechanisms for open- and closed-type X/A-like cells since open-type cells receive luminal signals such as nutrients while closed-type cells are stimulated by hormones, mechanical distension, or neuronal signals (Sakata, I. et al. 2010). As the second most abundant gastric endocrine cell type, the X/A-like cell makes up 20-30% of the oxyntic endocrine cells and works in conjunction with several other enteroendocrine cells to influence gastric acid secretion (Rindi, G. et al. 2004; Rindi, G. et al. 2004). Now commonly referred to as the ghrelin cell, the assessment of ghrelin production and secretion in X/A-like cells has attracted further studies. In Chapter 5 of my thesis, I will discuss my characterization of another enteroendocrine cell, the histaminergic ECL cell, and propose future studies pertaining to how ghrelin cells and ECL cells are interconnected within the gastric community.

The entire makeup of gastric enteroendocrine cells includes a large population of ECL cells that produce histamine, X/A-like cells that secrete ghrelin, D-cells that release somatostatin, G-cells containing gastrin, and fewer enterochromaffin (EC) cells containing serotonin (Simonsson, M. et al. 1988; Rindi, G. et al. 2004) (Figure 1.1). Other cell types in the stomach include hydrochloric acid (HCl) secreting parietal cells, zymogenic (chief) cells that produce pepsinogen, and mucous cells (Chu, S. et al. 2012). Interactions and paracrine signaling between these cell types are fundamental to the regulation of gastric acid secretion, facilitating the digestion of protein and preventing gastric infection (Chu, S. et al. 2012) (Figure

1.1). Different patterns of cell populations reside in discrete regions of the stomach, which can be divided into three anatomical regions consisting of the fundus, corpus/body, and antrum (Chu, S. et al. 2012) (Figure 1.1). Alternatively, the stomach can be characterized by two functional glandular regions, the oxyntic and pyloric mucosa, which are also unique in their display of cell of populations (Chu, S. et al. 2012). Parietal cells and ECL cells are prevailing cell-types in the oxyntic mucosa, although X/A-like cells, D cells, and EC cells also reside in this region (Chu, S. et al. 2012). The pyloric mucosa stands apart from the oxyntic mucosa through its display of G-cells (Chu, S. et al. 2012). Gastrin produced by G-cells stimulates gastric acid secretion from parietal cells and mucosal cell growth (Fourmy, D. et al. 2011). A significant part of gastrin's effects on gastric acid release is mediated through gastrin's activation of cholecystokinin-2 (CCK_2) receptors on ECL cells (Lindstrom, E. et al. 2001; Schubert, M.L. 2008; Waldum, H.L. et al. 2009; Chen, D. et al. 2010) (Figure 1.1). Gastrin accelerates histamine synthesis, production of secretory granules, and formation of secretory vesicles while simultaneously triggering exocytosis of docked vesicles in ECL cells (Chen, D. et al. 1994; Chen, D. et al. 1996; Chen, D. et al. 1996; Chen, D. et al. 1999). Chronic, long-term gastrin stimulation of ECL cells can lead to hypertrophy, hyperplasia, and ECLomas (Hakanson, R. et al. 1990; Chu, S. et al. 2012). Additionally, gastrin activates histidine decarboxylase (HDC) in ECL cells, which acts as a catalyzing enzyme in the formation of histamine (Zhao, C.M. et al. 2012). Histamine released from ECL cells binds directly to histamine H-2 receptors on parietal cells to instigate hydrochloric acid secretion, thus completing the gastrin cell-ECL cell-parietal cell axis (Chu, S. et al. 2012; Zhao, C.M. et al. 2012) (Figure 1.1). Analysis through electron microscopy has revealed that ECL cells display arborizations of extended neuronal-like cytoplasmic processes that end nearby parietal cells (Gustafsson, B.I. et al. 2011). Thus, release of ECL histamine provokes a rapid, vigorous response of parietal cell acid secretion. Findings from other studies led researchers to conceive that vagal nerve fibers containing pituitary adenylate cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP)

within close proximity to ECL cells are also responsible for stimulation and growth of these cells (Axelson, J. et al. 1988; Ekblad, E. et al. 2000; Norlen, P. et al. 2005). Of note, somatostatin (produced in D-cells) has been shown to inhibit ECL cell histamine secretion and proliferation (Prinz, C. et al. 1993; Lindstrom, E. et al. 1997) (Figure 1.1).

Amidst these factors that regulate gastric acid secretion, ghrelin was also found to have an effect on both gastric motility and acid secretion, although results have been inconsistent (Masuda, Y. et al. 2000; Date, Y. et al. 2001; Levin, F. et al. 2005). Using a lumen-perfused method of administration, ghrelin increased gastric acid secretion almost to the extent of histamine (Masuda, Y. et al. 2000). Ghrelin administration to the central nervous system dose-dependently increased gastric acid output and showed a synergistic effect with gastrin in stimulating acid secretion, but these effects were abolished by vagotomy (Date, Y. et al. 2001; Fukumoto, K. et al. 2008). On the other hand, peripherally administered ghrelin reduced pentagastrin-stimulated gastric acid secretion, but still increased gastric motility (Levin, F. et al. 2005). Another study reported no effect of ghrelin on gastric acid secretion (Dornonville de la Cour, C. et al. 2004). Variations in the method of ghrelin administration, rodent model used (mouse vs. rat), and state of the animal (anesthetized vs. awake) may account for these discrepancies.

In terms of central nervous system effects, it was primarily observed that ghrelin acts on GHSRs to stimulate GH release in the pituitary, and initial studies also detected effects of ghrelin on hypothalamic neurons, food intake, and energy homeostasis (Kamegai, J. et al. 2000; Masuda, Y. et al. 2000; Peino, R. et al. 2000; Takaya, K. et al. 2000; Tschop, M. et al. 2000; Wren, A.M. et al. 2000). The expression of *Ghsr* mRNA and ghrelin-induced neuronal activation was discovered to be high in hypothalamic regions, including the arcuate nucleus (ARC), lateral hypothalamus (LH), paraventricular nucleus (PVN), and dorsomedial nucleus (Lawrence, C.B.

et al. 2002; Zigman, J.M. et al. 2006). The combination of these findings led researchers to focus on the role of ghrelin in appetite and metabolism.

Ghrelin's role in homeostatic feeding and glucose metabolism

Numerous studies revealed orexigenic properties of ghrelin in both rodents and humans, demonstrating that it can stimulate and enhance food intake as well as increase meal size, resulting in obesity if levels are consistently elevated (Wren, A.M. et al. 2000; Wren, A.M. et al. 2001; Wren, A.M. et al. 2001; Lugar, C.W. et al. 2004). Ghrelin's orexigenic actions are transient, yet potent, and ghrelin elicits eating despite a state of satiation (Nakazato, M. et al. 2001; Wren, A.M. et al. 2001; Cummings, D.E. 2006). Furthermore, plasma ghrelin levels were found to have a daily rhythm corresponding with feeding schedules, showing sharp elevations before set meal times and lowering after each meal (Cummings, D.E. et al. 2001; Sugino, T. et al. 2002; Kalra, S.P. et al. 2003; Bodosi, B. et al. 2004). This rhythm could occur due to fluctuations in signals provided by nutrients in the gastrointestinal tract or variations in glucose levels (Williams, D.L. et al. 2005; Sakata, I. et al. 2012). Rodents typically display anticipatory elevations in locomotor activity prior to mealtimes, and the timing of preprandial spikes in ghrelin correlates with anticipatory hyperlocomotion (Drazen, D.L. et al. 2006). Ghrelin-receptor deficient mice have a reduced anticipatory locomotor response before scheduled meals, while pharmacological ghrelin administration stimulates locomotor activity and foraging behavior in rats and hamsters (Keen-Rhinehart, E. et al. 2005; Jerlhag, E. et al. 2007; Blum, I.D. et al. 2009). Collectively, these findings led to the hypothesis that meal anticipation prompts ghrelin secretion, resulting in an anticipatory arousal of locomotor activity (Blum, I.D. et al. 2009).

The dense expression of ghrelin receptors in the ARC led researchers to believe that ghrelin activates ARC neurons to stimulate hypothalamic pathways, resulting in an orexigenic response (Howard, A.D. et al. 1996; Guan, X.M. et al. 1997; Willesen, M.G. et al. 1999;

Nakazato, M. et al. 2001; Zigman, J.M. et al. 2006; Kageyama, H. et al. 2010). Indeed, ghrelin administration results in the expression of c-Fos protein, a marker of neuronal activation, in neuropeptide Y (NPY) neurons and agouti-related protein (AgRP) neurons in the ARC (Nakazato, M. et al. 2001). These NPY/AgRP neurons project to the LH and PVN, contributing to the interconnected network of hypothalamic pathways (Bouret, S.G. et al. 2004). Importantly, stimulation of NPY/AgRP neurons leads to a strong feeding response (Bouret, S.G. et al. 2004). Multiple studies have revealed that ghrelin prompts food intake by activation of GHSRs on NPY/AgRP neurons (Willesen, M.G. et al. 1999; Nakazato, M. et al. 2001; Wren, A.M. et al. 2001; Kageyama, H. et al. 2010; Schaeffer, M. et al. 2013). A recent study from the Zigman lab using a mouse model in which GHSR expression is present solely in AgRP neurons has shown the role of the AgRP neuronal population in ghrelin's orexigenic and glucoregulatory actions (Wang, Q. et al. 2014). Specifically, ghrelin signaling in AgRP neurons stimulated a significant, although partial, orexigenic response and was sufficient in protecting against lowered blood glucose levels observed with caloric restriction in *Ghsr*-null mice (Wang, Q. et al. 2014). However, ghrelin has the capability to stimulate food intake by stimulating other brain regions and neuronal types as well, such as neurons in the LH, PVN, or ventral tegmental area (VTA) (Olszewski, P.K. et al. 2003; Olszewski, P.K. et al. 2003; Toshinai, K. et al. 2003; Naleid, A.M. et al. 2005).

As mentioned briefly, ghrelin undergoes a post-translational modification in which the enzyme ghrelin-O-acyl transferase (GOAT) octanoylates a serine and transforms desacyl ghrelin to acyl-ghrelin (Yang, J. et al. 2008; Kojima, M. et al. 2010). This modification is necessary in order for ghrelin to bind to GHSRs, and a receptor for desacyl ghrelin has yet to be discovered (Yang, J. et al. 2008; Kojima, M. et al. 2010). In fact, the existence of a separate receptor for desacyl ghrelin is highly suspected due to studies showing effects of desacyl ghrelin on physiological responses such as food intake, glucose metabolism, and adiposity (Toshinai,

K. et al. 2006; Delhanty, P.J. et al. 2012; Delhanty, P.J. et al. 2013; Delhanty, P.J. et al. 2013). Future studies will likely elaborate on the importance of desacyl ghrelin in homeostatic feeding and body weight regulation.

Extensive studies have also been performed studying ghrelin's role in homeostatic feeding using transgenic mouse models and gene-targeted knock-in/knockout mouse models (Uchida, A. et al. 2013; Mason, B.L. et al. 2014). A transgenic model of ghrelin overexpression using a bacterial artificial chromosome containing the ghrelin gene and its promotor revealed increased food intake without a significant change in body weight, attributed to increased energy expenditure (Bewick, G.A. et al. 2009). On the contrary, overexpression of ghrelin in the brain using the neuron-specific enolase promotor produced a 5-fold increase in circulating ghrelin, but did not affect energy expenditure, body weight, fat mass, or food intake (Reed, J.A. et al. 2008). It is possible that elevating ghrelin to such an extent in this mouse model results in the internalization of ghrelin receptors, leading to ghrelin resistance and the lack of a distinct phenotype. Surprisingly, ghrelin knockout mice exhibit no change in body weight or food intake under a standard chow diet, despite having a complete lack of circulating ghrelin (Sun, Y. et al. 2003; Wortley, K.E. et al. 2004; De Smet, B. et al. 2006; Dezaki, K. et al. 2006). Under chronic high fat diet (HFD) feeding, mice with ghrelin deficiency displayed lower body weight and fat mass as compared to wild-type mice when exposed to HFD at an early age, but not when exposed to HFD at an older age (Wortley, K.E. et al. 2004; Wortley, K.E. et al. 2005; Sun, Y. et al. 2008). However, feeding performance in all models of ghrelin deficiency revealed a lack of an effect on homeostatic aspects of feeding, such as cumulative feeding, post-fasting hyperphagia, and memory-related feeding (Sun, Y. et al. 2003; Wortley, K.E. et al. 2004; De Smet, B. et al. 2006; Pfluger, P.T. et al. 2008; Sato, T. et al. 2008). Therefore, it is likely that the lack of ghrelin during development in these knockout mouse models drove compensatory changes to offset the loss of ghrelin.

Interestingly, mouse models lacking GHSR expression yield differing results from ghrelin knockout mice. Although GHSR deficient mice do not display differences in food intake under a standard chow diet, they do have significantly reduced body weights (Sun, Y. et al. 2004; Zigman, J.M. et al. 2005). The Zigman lab has reported that female *Ghsr*-null mice fed on a standard chow diet have reduced body weight as compared to wild-type mice, starting at 12 weeks of age (Zigman, J.M. et al. 2005). These *Ghsr*-null mice have a modification in the *Ghsr* locus involving the insertion of a transcriptional blocking cassette flanked by lox-P sites. As a result, *Ghsr*-null mice lack functional *Ghsr* expression but maintain the ability to have region-specific restoration of *Ghsr* expression using Cre-recombinase. Utilizing this mouse model, specific rescue of *Ghsr* expression in chatecholaminergic neurons was sufficient to partially mediate ghrelin's orexigenic effects, while rescue of *Ghsr* expression in specific hindbrain nuclei did not reinstate ghrelin-induced feeding (Chuang, J.C. et al. 2011; Scott, M.M. et al. 2012). Upon early exposure to HFD, *Ghsr*-null mice are resistant to HFD-induced weight gain and adiposity (Zigman, J.M. et al. 2005). GHSR knockout mice were also found to be lighter than wild-type mice during the ages of 16-24 weeks (Sun, Y. et al. 2004). In response to repeated fasts, GHSR knockout mice do not increase their food intake as observed in wild-type mice (Abizaid, A. et al. 2006), and they exhibit a reduced level of age-associated obesity (Lin, L. et al. 2011; Ma, X. et al. 2011).

The differences between ghrelin deficient models and GHSR deficient models highlight the notion that GHSRs may have activity independent of ghrelin mediation. Indeed, assessment of ghrelin receptors has revealed constitutive receptor activity, heterodimer formation, and/or functional interaction with other G-protein coupled receptors (Jiang, H. et al. 2006; Damian, M. et al. 2012; Kern, A. et al. 2012; Park, S. et al. 2012). Furthermore, in the instance of both ghrelin and ghrelin receptor deficiency in a double knockout mouse model, a significant reduction in body weight and increase in energy expenditure was observed under the standard

chow diet, while single ghrelin or GHSR knockout models did not display any effects on energy balance (Pfluger, P.T. et al. 2008). This finding suggests that additional factors and molecular mediators may be involved in ghrelin signaling.

Ghrelin may also indirectly affect body weight and feeding behavior through regulation of whole-body glucose homeostasis. Several studies have demonstrated a relationship between ghrelin, glucose, and insulin (Dezaki, K. et al. 2004; Dezaki, K. et al. 2006; Dezaki, K. et al. 2007; Chuang, J.C. et al. 2011). Insulin is vital for cellular-uptake of glucose, and improper functioning or lack of insulin can lead to hyperglycemia and diabetes. Excessive long-term elevations in blood glucose, as occurs with diabetes, can be quite harmful to an individual's health, often resulting in damage to blood vessels, cardiovascular disease, and kidney disease, among others (O'Gara, P.T. et al. 2013). On the other hand, hypoglycemia can be fatal as well, causing seizures, unconsciousness, brain damage, and death (Shin, B.S. et al. 2010; Zoungas, S. et al. 2010). Hence, proper regulation of glucose metabolism is vital for cell functioning and overall health, so factors that regulate glucose and/or insulin levels may provide insight to new methods for treating dangerous variations in blood sugar. Low ambient d-glucose concentrations were found to stimulate ghrelin release, while high d-glucose concentrations or glucose metabolism inhibited ghrelin release (Sakata, I. et al. 2012).

Regarding the relationship of ghrelin to glucose, ghrelin administration in rodents dose-dependently increases fasting blood glucose, reduces insulin levels, and diminishes insulin responses during glucose tolerance testing (Dezaki, K. et al. 2004; Dezaki, K. et al. 2007). Studies performed in isolated rodent pancreatic islets, ghrelin-overexpressing mice, and humans have yielded similar results, displaying ghrelin-mediated increases of glucose and reductions of insulin release (Broglio, F. et al. 2001; Colombo, M. et al. 2003; Dezaki, K. et al. 2004; Dezaki, K. et al. 2007; Reed, J.A. et al. 2008). Ghrelin deletion improves glucose

tolerance and elevates glucose-stimulated insulin secreted from isolated islets, while ghrelin receptor deletion correspondingly enhances insulin sensitivity and lowers blood glucose (Zigman, J.M. et al. 2005; Dezaki, K. et al. 2006; Dezaki, K. et al. 2007; Longo, K.A. et al. 2008; Qi, Y. et al. 2011). Emphasizing the importance of ghrelin in glucose metabolism, calorically restricted GOAT-deficient mice show a nearly fatal decline in fasting blood glucose after only 7 days (Zhao, T.J. et al. 2010). These low levels can be normalized upon acyl-ghrelin or GH administration, again stressing the role of ghrelin in glucose maintenance (Zhao, T.J. et al. 2010). Typically, a normal response to hypoglycemia would be increased glucagon secretion, which in turn releases glucose into the blood from glycogen stores in the liver.

A thorough study from the Zigman lab demonstrated that ghrelin directly stimulates pancreatic α-cells to secrete glucagon (Chuang, J.C. et al. 2011). Ghrelin administration enhanced glucagon and glucose plasma levels in mice and stimulated glucagon secretion in isolated islets as well as pancreatic α-cell lines (Chuang, J.C. et al. 2011). *Ghsr* expression was highly enriched in these pancreatic α-cells, and mice lacking ghrelin receptors exhibited lower plasma glucagon and glucose (Chuang, J.C. et al. 2011). Lastly, ghrelin-mediated glucagon secretion in pancreatic α-cells was dependent upon phosphorylation of extracellular regulated kinases (ERK) (Chuang, J.C. et al. 2011). Overall, these findings provide evidence that ghrelin-induced elevations in circulating blood glucose are in part due to its stimulation of glucagon secretion. Studying the secretion of metabolic regulators such as glucagon advances our understanding of the pathways involved in maintaining metabolic homeostasis. In Chapter 4 of my thesis, I will explore ghrelin cell expression and secretion of alternate proteins that may also be involved in metabolic regulatory pathways. These experiments reveal ghrelin cell expression and secretion of RBP4, which is a protein that has been identified as an important signal for insulin resistance, and Chapter 4 elaborates on the significance of these findings (Graham, T.E. et al. 2007).

To summarize these historical studies, under normal, non-stressed conditions, ghrelin signaling pathways serve as important mediators of homeostatic feeding, body weight regulation, and glucose metabolism (Kojima, M. et al. 1999; Nakazato, M. et al. 2001; Dezaki, K. et al. 2004; Gil-Campos, M. et al. 2006; Chuang, J.C. et al. 2011). As the only gut-produced peptide that has orexigenic effects, ghrelin is unique among the assortment of other feeding peptides (cholecystokinin, peptide YY, glucagon-like peptide, leptin, etc.) and remains an important topic of study today (Skibicka, K.P. et al. 2011).

Ghrelin's role in more complex, hedonic feeding behaviors

The obesity epidemic: a struggle between homeostatic feeding and hedonic feeding

Ghrelin signaling works in combination with other hormones and neuropeptides in a complex network that regulates feeding, metabolism, and proper energy balance (Kojima, M. et al. 1999; Nakazato, M. et al. 2001; Gil-Campos, M. et al. 2006; Klok, M.D. et al. 2007). Controlling the amount of energy intake based on the amount of energy expenditure through this network of signaling is crucial in order to prevent weight problems such as obesity (Klok, M.D. et al. 2007). Given that over one third (34.9%) of adults in the United States were obese in 2011-2012 and that serious health risks are associated with obesity, understanding the peripheral signals and neurochemical pathways involved in the regulation of feeding is crucial (Ogden et al. 2013). Classically, brain circuits in the hypothalamus and brain stem were characterized as the main control centers of food intake, but more recent studies have provided evidence for a multi-process integrated mechanism of feeding which includes regions of the brain involved in learning and reward related behaviors, such as the hippocampus, amygdala, prefrontal cortex (PFC), ventral tegmental area (VTA), and nucleus accumbens (NAcc) (Petrovich, G.D. et al. 2005; Petrovich, G.D. et al. 2007; Dickson, S.L. et al. 2011; Skibicka, K.P.

et al. 2011; Volkow, N.D. et al. 2011; Skibicka, K.P. et al. 2012). Therefore, overconsumption may result from the stimulation of circuitry involved in food reward, which in turn overrides normal homeostatic feeding circuitry and satiety signals (Volkow, N.D. et al. 2008; Volkow, N.D. et al. 2011). To that end, it is of high priority to study hedonic feeding behavior since multiple factors, both environmental and genetic, can disturb normal homeostatic feeding processes and lead to the excessive intake of food, including increases in the consumption of pleasurable foods high in sugar and fat (Weingarten, H.P. 1983; Petrovich, G.D. et al. 2007; Volkow, N.D. et al. 2008).

Pharmacologic manipulations in ghrelin and food reward

Ghrelin plays a significant role in food reward behavior and hedonic feeding, particularly when homeostatic ghrelin signaling is disturbed through abnormal elevations or when ghrelin rhythms are disrupted (Perello, M. et al. 2010; Davis, J.F. et al. 2012; Landgren, S. et al. 2012; Perello, M. et al. 2012). For example, intracerebroventricular administration of ghrelin in rodents shifts food preference towards diets high in fat (Tschop, M. et al. 2000; Shimbara, T. et al. 2004), while peripheral ghrelin administration increases consumption and preference of saccharin flavored foods in wild-type mice but not in GHSR-deficient littermates. Administration of a GHSR antagonist lowers self-administration of saccharin solution and steers preference away from a sucrose solution and towards water in a two-bottle choice drinking study (Landgren, S. et al. 2011). In a free choice protocol, the consumption of regular chow is not reduced in GHSR-deficient mice or mice administered a GHSR antagonist, while the consumption of peanut butter is significantly reduced (Egecioglu, E. et al. 2010). Previous studies performed by the Zigman lab demonstrated ghrelin's involvement in conditioned place preference (CPP) for HFD (Perello, M. et al. 2010). In this behavioral protocol, mice are paired with HFD in one chamber and on alternating days, paired with regular chow in a second

chamber. The chambers are composed of distinct visual and tactile cues (wall patterns and flooring) so that the two can be easily distinguished from one another. On the test day, mice are allowed free access to both chambers, and the time spent in each is recorded. A significant increase in the amount of time spent in the HFD-paired chamber after conditioning as compared to a baseline pretest denotes the development of CPP, a conditioned preference for a particular chamber. Ghrelin administration prior to each conditioning session, but not on the actual test day, was sufficient to generate CPP for HFD (Perello, M. et al. 2010). In addition, ghrelin administration solely on the test day, about 20 minutes prior to testing, also induced CPP for HFD (Perello, M. et al. 2010). The timing of ghrelin administration is key because it indicates that ghrelin can mediate the acquisition of CPP, during the learning or conditioning phase, as well as the expression of CPP during testing. Pretreatment with an orexin receptor 1 selective antagonist, SB334867, prior to ghrelin administration blocked the development of CPP, although it did not affect ghrelin's orexigenic properties (Perello, M. et al. 2010). Orexin-deficient mice also failed to acquire CPP for HFD, suggesting that ghrelin regulates CPP for HFD through orexinergic pathways (Perello, M. et al. 2010). Dissecting this food reward behavior from basic feeding responses, orexinergic neurons did not mediate ghrelin's stimulation of food intake, as shown by the maintenance of ghrelin's orexigenic response despite administration of SB334867 (Perello, M. et al. 2010).

Work performed in this thesis (Chapter 3) revealed that intact ghrelin signaling is required for normal development of cue-potentiated feeding, a behavior that stimulates food intake through a learned food-cue association, as shown through pharmacological and genetic blockade of GHSRs (Walker, A.K. et al. 2012). In other studies, ghrelin administration enhanced both cue-potentiated feeding responses and Pavlovian-Instrumental Transfer, suggesting a strengthened ability to associate cues with food and craving (Johnson, A.W. et al.

2009; Kanoski, S.E. et al. 2013). The operant conditioning paradigm, which aims at determining motivation to work for a food reward, is also affected by ghrelin. Operant lever pressing/nose poking for HFD pellets, sucrose, and peanut butter flavored sucrose was significantly increased by ghrelin, while a GHSR antagonist reduced lever pressing for sucrose (Perello, M. et al. 2010; Skibicka, K.P. et al. 2011; Finger, B.C. et al. 2012; Skibicka, K.P. et al. 2012; Kanoski, S.E. et al. 2013). Treatment with SB334867 prior to ghrelin administration blocked enhanced operant responding for HFD, suggesting that ghrelin acts through orexin neurons to increase motivated food reward behaviors (Perello, M. et al. 2010). Ghrelin's effects on both operant conditioning for sucrose and cue-potentiated feeding can also be mediated by ghrelin signaling in the ventral hippocampus (Kanoski, S.E. et al. 2013). Ghrelin receptors are expressed in regions of the hippocampus as well as the amygdala, both of which may contribute to the learning aspect of food reward or habitual feeding behavior (Figure 1.2A)(Guan, X.M. et al. 1997; Zigman, J.M. et al. 2006; Walker, A.K. et al. 2012).

Endogenous elevations in ghrelin and food reward

Certain external stressors or states of energy insufficiency can lead to organic elevations in ghrelin, which may have more relevance to behavior than artificial elevations by pharmacologic means. Factors that affect ghrelin signaling through endogenous ghrelin elevations include caloric restriction (overnight fasting or chronic restriction), acute stress (tail pinch, tail suspension), and chronic stress (chronic social defeat stress [CSDS], chronic unpredictable stress) (Asakawa, A. et al. 2001; Gold, P.W. et al. 2002; Kristenssson, E. et al. 2006; de la Cour, C.D. et al. 2007; Rouach, V. et al. 2007; Lutter, M. et al. 2008; Ochi, M. et al. 2008). In the instance of food deprivation, elevations in ghrelin regulate behaviors related to food reward that have a strong learning component such as hunger- and cue-induced appetitive behavior (Davidson, T.L. et al. 2005; Jewett, D.C. et al. 2006). CPP for pleasurable foods can

be instigated by endogenous elevations in ghrelin (via caloric restriction or chronic stress) as well as through pharmacologic administration of ghrelin; this development of CPP is blocked upon administration of an orexin receptor antagonist during the conditioning sessions or through genetic blockade in *Ghsr*-null mice (Perello, M. et al. 2010; Chuang, J.C. et al. 2011; Disse, E. et al. 2011). CSDS also elevates total daily food intake by increasing meal size, and CSDS significantly increases intake of high fat diet (Chuang, J.C. et al. 2011; Kumar, J. et al. 2013).

Ghrelin in human body weight regulation and feeding behavior

Ghrelin stimulates human appetite

Human studies elaborate upon those performed in mice to implicate ghrelin in the regulation of homeostatic and hedonic feeding. Peaks in pre-prandial ghrelin occur prior to mealtimes in human subjects on a fixed feeding schedule, and food consumption increases with ghrelin administration (Cummings, D.E. et al. 2001; Cummings, D.E. 2006). Ghrelin's potent effects on meal initiation and food intake in humans highlights the necessity of understanding every aspect of ghrelin signaling, as it may one day prove to be a target for obesity treatments.

Involvement of ghrelin signaling in human body weight regulation

In terms of obesity and body weight regulation, circulating ghrelin levels are actually lower in most obese patients compared to normal subjects, suggesting that obese individuals may be more sensitive to spikes in ghrelin due to a lower baseline level (Marchesini *et al.*, 2004; Nagaya *et al.*, 2001; Shimizu *et al.*, 2003; Tschop *et al.*, 2001b). Along with a lower baseline level of ghrelin, normal ghrelin rhythms are disturbed in obese individuals, as shown by blunted, flat nocturnal levels (Yildiz, B.O. et al. 2004). Of note, studies from another group show that diet-induced obesity in mice results in ghrelin resistance that can be reversed upon caloric

restriction (Briggs, D.I. et al. 2013). Therefore, an alternate hypothesis is that a reversal of ghrelin resistance may occur with dieting that results in rebound weight gain (Briggs, D.I. et al. 2013). The appropriate ghrelin response to food intake is altered in obese children. Three hours after food intake, ghrelin levels of obese children recover and increase towards baseline levels, whereas ghrelin remains suppressed in normal weight children (Schellekens, H. et al. 2010). Therefore, there may be a shorter period of time after each meal that an obese child feels full and satisfied, leading to snacking or the sensation of hunger faster than normal weight children. Strong connections between obesity and human genetic mutations related to aberrant ghrelin signaling also exist. Nucleotide changes in the preproghrelin locus and rare mutations in the *Ghsr* gene have been associated with obesity in humans (Ukkola, O. et al. 2001; Hinney, A. et al. 2002; Liu, G. et al. 2007). However, not all mutations in the *Ghsr* gene result in obesity, revealing that the how the mutation specifically affects the receptor function is an important element in determining how it affects weight regulation (Pantel, J. et al. 2006).

As opposed to the more common diet induced obesity, abnormally high ghrelin levels may contribute to the excessive hyperphagia and life-threatening obesity that occurs with the genetic disorder, Prader-Willi Syndrome (Cummings, D.E. et al. 2002; DelParigi, A. et al. 2002; Tauber, M. et al. 2004). In fact, prior to the development of hyperphagia and weight gain, ghrelin levels in young children with Prader-Willi Syndrome do not differ from control subjects, correlating the appearance of these symptoms with the onset of elevated ghrelin levels (Erdie-Lalena, C.R. et al. 2006). Ghrelin levels are elevated in dieting individuals who have achieved weight loss; these elevations may influence the rebound weight gain often that often occurs with the dieting process (Cummings, D.E. et al. 2002; Reinehr, T. et al. 2005). Alternatively, a more successful method of prolonged weight loss, the Roux-en-Y gastric bypass (RYGB) surgery, may attribute its effectiveness to diminished ghrelin levels that were found to have a greater

than 70% reduction as compared to obese counterparts (Cummings, D.E. et al. 2002). Ghrelin levels are reduced compared to baseline levels both thirty minutes after RYGB surgery and after weight loss occurs from RYGB surgery (Lin, E. et al. 2004; Morinigo, R. et al. 2004; Beckman, L.M. et al. 2010).

A new current study has discerned a link connecting ghrelin with the fat mass and obesity-associated protein, FTO (Karra, E. et al. 2013). The *FTO* gene encodes a 2-oxoglutarate dependent nucleic acid demethylase and shows a high similarity with the enzyme AlkB, which demethylates DNA (Gerken, T. et al. 2007; Jia, G. et al. 2011). FTO has been shown to modify RNA by demethylation of N^6 methyladenosine, although the exact regulatory effect of this modification remains unknown (Gerken, T. et al. 2007; Jia, G. et al. 2011). Of particular importance, polymorphisms in *FTO* are consistently associated with human obesity, increased food intake, and significant consumption of calorically dense foods (Karra, E. et al 2013). Across a wide range of ages and populations, genome-wide association studies have revealed a strong correlation between single nucleotide polymorphisms (SNPs) in the first intron of *FTO* and elevated body mass index (BMI) and adiposity (Dina, C. et al. 2007; Frayling, T.M. et al. 2007; Hardy, R. et al. 2010; Li, H. et al. 2012; Yang, J. et al. 2012; Zhou, D. et al. 2012).

An example of one such polymorphism is the “obesity risk” A allele of *FTO* in which homozygous AA individuals display increased energy intake, with a preference for fat consumption, and reduced satiety leads to increased obesity (Cecil, J.E. et al. 2008; Speakman, J.R. et al. 2008; Timpson, N.J. et al. 2008; Wardle, J. et al. 2008; Tanofsky-Kraff, M. et al. 2009). Weight matched individuals homozygous for the A allele or the low risk T allele of *FTO* were given meals with equal caloric loads, and postprandial ghrelin levels were measured (Karra, E. et al. 2013). AA subjects failed to suppress ghrelin appropriately after meal

consumption, and they also reported attenuated levels of satiety as compared to TT subjects (Karra, E. et al. 2013). After meal consumption, AA subjects rated images of high calorie foods as significantly more appealing than TT subjects, and brain imaging studies revealed a significant effect of the *FTO* genotype on neural responses to food images (Karra, E. et al. 2013). In the fasted state, AA and TT subjects exhibit differential neural responses to ghrelin in the nucleus accumbens (NAc), hypothalamus, thalamus, orbitofrontal cortex, parahippocampus, and cingulate. In these regions, fasted TT subjects have increased neural responses with greater levels of ghrelin, while AA subjects display reduced neural responses (Karra, E. et al. 2013). Postprandial responses to food images were also affected in the prefrontal cortex (PFC) (Ott, V. et al. 2014). Therefore, the *FTO* genotype influences how different regions in our brain react to food cues and interacts with ghrelin to regulate neural responses. Furthermore, FTO knockout mice show a reduction in acyl ghrelin/total ghrelin ratios, while overexpression of *FTO* in cell culture increased *Ghrl* (gene for ghrelin) and *Goat* mRNA as well as acyl-ghrelin/total ghrelin ratios (Karra, E. et al. 2013). Blood cells from AA subjects revealed increased *Ghrl* mRNA, reduced m^6A methylation of *Ghrl* mRNA, and increased *FTO* mRNA as compared to TT subjects, suggesting this polymorphism is a gain-of-function mutation that regulates ghrelin mRNA expression (Karra, E. et al. 2013). Overall, FTO's regulation of *Ghrl* mRNA expression could have substantial effects on food intake, food preference, body weight, and adiposity providing evidence behind the obese phenotype in individuals with *FTO* polymorphisms.

Ghrelin regulates human food reward behavior

Shifting toward a hedonic aspect of human feeding, ghrelin influences the response to food cues and the rewarding value of food. One human imaging study revealed that administering ghrelin to subjects increases activity in brain regions involved in assessing reward value related to food and food cues, including the amygdala, striatum, orbitofrontal cortex, and

anterior insula, but not in the homeostatic hypothalamus (Malik, S. et al. 2008). Ghrelin administration increases the amount of money subjects are willing to spend on food and its perceived value, while decreasing the value of non-food objects (Tang, 2011). Complementing earlier experiments performed in rodent stress models, human tests of psychosocial stress induce elevations in plasma ghrelin, potentially contributing to the stress-eating response of high fat diet intake commonly observed in human behavior. (Mokdad, A.H. et al. 2000; Dallman, M.F. et al. 2003; Raspopow, K. et al. 2010). Additionally, ghrelin levels decline after food intake in stressed subjects characterized as non-emotional eaters, while ghrelin levels were unaffected in stressed subjects characterized as emotional eaters (Raspopow, K. et al. 2010). The lack of a normalization to baseline ghrelin levels in emotional eaters correlates with their increased food consumption in response to stress (Raspopow, K. et al. 2010). Taken all together, ghrelin plays a pertinent role in controlling typical day-to-day feeding, body weight regulation, and the development of more complex food reward behaviors. In Chapter 3 of my thesis, I will discuss the role of ghrelin in the cue-potentiated feeding paradigm, which is a behavior that reflects habitual eating.

Ghrelin as a mediator of dopaminergic signaling in reward pathway

Ghrelin can regulate feeding behavior through the VTA

As ghrelin's role in hedonic feeding became increasingly clear, two primary findings led to the hypothesis that neurons in the VTA play an important role in ghrelin mediated food reward. The demonstration of high *Ghsr* mRNA expression in the VTA and the discovery of strong feeding effects after ghrelin microinjection into the VTA paved the way for studies of food reward behavior (Guan, X.M. et al. 1997; Naleid, A.M. et al. 2005; Zigman, J.M. et al. 2006). Concentrating on a more hedonic aspect of feeding, it was revealed that chemical lesions of the

VTA abolish the selective increase in palatable food that occurs with central ghrelin administration without affecting normal chow intake (Egecioglu, E. et al. 2010). This provided a strong argument for the idea that ghrelin action specifically in the VTA is responsible for the elevated intake of rewarding foods and suggested potential mechanism for ghrelin in more complex food reward behaviors. Indeed, it has now been established that catecholaminergic neurons largely mediate the effects of stress-induced ghrelin on CPP, one model of food reward behavior (Chuang, J.C. et al. 2011). To summarize the study, re-expression of ghrelin receptors in cells containing tyrosine-hydroxylase (TH) significantly restores stress-induced CPP for HFD lacking in *Ghsr*-null mice (Chuang, J.C. et al. 2011). Ghrelin receptor expression specifically in catecholaminergic neurons also rescued CPP for HFD induced by ghrelin administration (Chuang, J.C. et al. 2011). Since the VTA contains a substantial population of dopamine neurons implicated in reward related behaviors, the next question became, "how does ghrelin affect dopamine signaling?"

Ghrelin enhances dopamine release

Ghrelin's influence on food reward behavior may be partially through its modulation of dopamine release into the striatum. Ghrelin elevates NAc dopamine levels after central or VTA-targeted ghrelin administration (Jerlhag, E. et al. 2006; Jerlhag, E. et al. 2007). Ghrelin also increases peak dopamine spikes in the NAc of rats eating palatable foods and increases nicotine-induced dopamine release as well as dopamine release into the amygdala in response to electrical stimulation (Kawahara, Y. et al. 2013; Palotai, M. et al. 2013; Palotai, M. et al. 2013). Even cues predictive of a food reward stimulate dopamine release in the NAc core and dorsomedial striatum (Brown, H.D. et al. 2011; McCutcheon, J.E. et al. 2012). Ghrelin's effects on dopamine release have been attributed to nicotinic receptor activation in central cholinergic pathways as well as through opioid receptors in the VTA by studies using receptor specific

antagonists that block ghrelin-induced dopamine release (Jerlhag, E. et al. 2006; Kawahara, Y. et al. 2013). Greater dopamine release from the VTA into the NAc, particularly during the ingestion of palatable foods, may contribute to the elevated incentive for and perceived value of those foods along with food cues (Jerlhag, E. et al. 2006).

Interaction of ghrelin receptors and dopamine receptors

Not only does ghrelin affect dopamine pathways through the modulation of alternate signaling (cholinergic, opioid pathway) that affects dopamine release in the striatum, but ghrelin signaling also has an interactive relationship with dopamine receptors. Through heterodimerization, the ghrelin receptor and dopamine receptor subtype 2 (DRD2) interact allosterically to modify normal dopamine receptor signaling (Kern, A. et al. 2012). Specifically, the DRD2 selective agonist quinpirole (as well as dopamine) dose-dependently increases $[Ca^{2+}]_i$ in cells co-expressing GHSR1a and DRD2, but not in cells expressing either GHSR1a or DRD2 alone (Kern, A. et al. 2012). Applying a GHSR antagonist to these cells attenuates the dopamine-induced Ca^{2+} mobilization (Kern, A. et al. 2012). Another strong DRD2 agonist, cabergoline, suppresses feeding in wild-type mice, but not Ghsr -/- mice, and administration of a ghrelin receptor antagonist prior to cabergoline blocks the suppression of feeding in wild-type mice (Kern, A. et al. 2012). Similarly, ghrelin receptors form heterodimers with dopamine receptor subtype 1 (DRD1), and cross-talk between these receptors amplifies DRD1 signaling (Jiang, H. et al. 2006). Conversely, dopamine receptor activity influences the behavioral effects of ghrelin. Central administration of dopamine receptor subtypes D1, D2, and D3 antagonists reduces ghrelin's orexigenic actions, while stimulation of dopamine receptor 1 singly, or dopamine receptors 1 and 2 simultaneously also reduces ghrelin's orexigenic actions (Romero-Pico, A. et al. 2013). Notably, the existence of heterodimer formations between ghrelin receptors and dopamine receptors raises an interesting point about mouse models used to

study ghrelin signaling. Specifically, discrepancies may arise when using ghrelin knockout mouse models and GHSR knockout mouse models, which may be due to ghrelin receptor functions that are independent of ghrelin binding to the receptor. Similarly, the use of ghrelin receptor antagonists, which prevent the binding of ghrelin to its receptor but do not block the interaction between receptors, may yield different results than the use of mouse models lacking ghrelin receptor expression. The use of both ghrelin knockout mice and GHSR knockout mice would be helpful in determining whether ghrelin is truly involved in a behavior or whether it is some function of the ghrelin receptor outside of ghrelin activation. Therefore, it is important to keep in mind that there are multiple aspects to ghrelin signaling (constitutive ghrelin receptor activity, ghrelin binding to the receptor resulting in downstream signaling cascades, and allosteric interactions between ghrelin receptors and other G protein-coupled receptors) that could separately contribute to functional outputs.

Overall, a comprehensive collection of studies have been performed which support a role for ghrelin's mediation of dopamine signaling, although further studies are required to outline the multiple neurochemical mechanisms that ghrelin influences in reward pathways. Moreover, limited studies have been performed focusing on ghrelin impact on different brain regions such as the hippocampus or amygdala.

The influence of ghrelin on mood

Interestingly, besides enhancing food intake, a multitude of studies using mice show that ghrelin also plays a critical role in modulating mood and an assortment of reward-related behaviors, particularly with chronic stress (Shimbara, T. et al. 2004; Lutter, M. et al. 2008; Malik, S. et al. 2008; Nakashima, K. et al. 2008; Schanze, A. et al. 2008; Barim, A.O. et al. 2009;

Lutter, M. et al. 2009; Egecioglu, E. et al. 2010; Perello, M. et al. 2010; Chuang, J.C. et al. 2011; Hansson, C. et al. 2011; King, S.J. et al. 2011; Kluge, M. et al. 2011; Landgren, S. et al. 2011; Steiger, A. et al. 2011; Skibicka, K.P. et al. 2012; Skibicka, K.P. et al. 2012). The first study showing ghrelin's antidepressant effects involved manipulations of ghrelin and determining time spent immobile in the forced swim test (FST), which is a commonly used test for antidepressant efficacy. Pharmacological ghrelin administration to wild-type mice reduces the amount of time spent immobile in the FST, indicating an antidepressant effect (Lutter, M. et al. 2008). Alternative means of elevating endogenous ghrelin levels, such as caloric restriction, revealed antidepressant-like effects in the FST with wild-type mice, but not *Ghsr*-null littermates (Lutter, M. et al. 2008). Ghrelin administration also reversed depressive-behavior induced by olfactory bulbectomy in mice (Carlini, V.P. et al. 2012). Studies of ghrelin and mood regulation suggest that ghrelin may be acting in the amygdala and the VTA to influence mood, and I have proposed that the hippocampus is likely involved as well (Figure 1.2B). Furthermore, human studies support the regulation of mood by ghrelin. In one study, ghrelin levels were discovered to be lower in depressed patients as compared to a control group, and a *Ghsr* polymorphism was found to be associated with major depressive disorder (Nakashima, K. et al. 2008; Barim, A.O. et al. 2009; Kluge, M. et al. 2011). Ghrelin treatments also reduced depressive symptoms in male patients suffering from major depressive disorder (Kluge, M. et al. 2011).

Notably, stress seems to be an important stimulus linking ghrelin to its modulation of mood, as external stress is widely acknowledged as a precipitating factor of depression and is known to significantly enhance ghrelin secretion (Asakawa, A. et al. 2001; Gold, P.W. et al. 2002; Kristenssson, E. et al. 2006; de la Cour, C.D. et al. 2007; Rouach, V. et al. 2007; Ochi, M. et al. 2008). Chronic stress also disrupts the natural circadian rhythm of ghrelin, with normalization of ghrelin cycles occuring after fluoxetine treatment (Kumar, J. et al. 2013). Of

particular importance, a prolonged increase in acyl-ghrelin levels in C57BL6/J mice occurs following CSDS (Lutter, M. et al. 2008). A collection of studies suggest that the stress-induced increase in ghrelin helps to minimize depressive-like behaviors and protect against anhedonia, as *Ghsr*-null mice, which cannot respond to increases in acyl-ghrelin, demonstrate exacerbated depression-like behaviors and lack CPP for HFD after CSDS (Lutter, M. et al. 2008; Chuang, J.C. et al. 2011). After CSDS, *Ghsr*-null mice spend significantly less time in the social interaction zone compared to wild-type littermates, reflecting a more severe display of depressive-like behavior (Lutter, M. et al. 2008). Restoring *Ghsr* expression in catcholaminergic neurons was sufficient to partially rescue the severe depressive phenotype observed in *Ghsr*-null mice (Chuang, J.C. et al. 2011). In the Chapter 6 discussion, I will elaborate on the significance and background of using CSDS as a behavioral model for depression.

Ghrelin's downstream effects on mood might be due to its neuroproliferative and neuroprotective characteristics, as ghrelin has been shown to protect multiple cell types against toxicity and enhance proliferation of neural progenitor cells in the dentate gyrus (DG) of the adult hippocampus (Chung, H. et al. 2007; Chung, H. et al. 2008; Johansson, I. et al. 2008; Andrews, Z.B. et al. 2009; Moon, M. et al. 2009; Moon, M. et al. 2009; Lee, J.Y. et al. 2010; Chung, H. et al. 2011; Lim, E. et al. 2011). Recently, ghrelin has also been suggested as having therapeutic potential for diseases involving debilitating neuronal degeneration, such as Alzheimer's or Parkinson's disease (Dos Santos, V.V. et al. 2013). In the setting of chronic stress, elevations in ghrelin may provide some level of protection against the reduction of neuronal proliferation and increase of cell death that occurs in the DG as a result of stress. Of note, the DG is one of two well characterized regions in the adult brain where neurogenesis occurs, and the neurogenic population is particularly susceptible to the harmful effects of external stressors (Altman, J. 1963; Reynolds, B.A. et al. 1992; Eriksson, P.S. et al. 1998).

In addition, a large body of work has identified the hippocampus as being involved in several aspects of depression, including that associated with reward-based learning; this was found to be through direct activity of ghrelin in the hippocampus or mediated by indirect connections with other brain regions (Sapolsky, R.M. 2001; Videbech, P. et al. 2004; Marsh, R. et al. 2010; Okatan, M. 2010; Luo, A.H. et al. 2011; MacQueen, G. et al. 2011; Frodl, T. et al. 2012). Given the neuroproliferative and neuroprotective effects of ghrelin, it is possible that ghrelin mediates mood through its influence on DG neurogenesis.

Neurogenesis and depression

Traditionally, the concept of neurogenesis was believed to pertain solely to embryonic and perinatal development in mammals (Ming, G.L. et al. 2005). Evidence for the development of new neurons in the adult hippocampus of rats broke the mold of traditional thinking and inspired further research into postnatal neurogenesis (Altman, J. et al. 1965). Many scientists remained skeptical whether adult neurogenesis was relevant to humans until a significant study revealed that humans display continuous neurogenesis throughout their lifespans (Eriksson, P.S. et al. 1998). It has now been well established that adult neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) in the lateral ventricles (LV) of the brain (Figure 1.3A). Interestingly, stem cells in the SVZ and SGZ produce cells that mature into different neuronal subtypes (Ming, G.L. et al. 2011). In the adult SVZ, neuroblasts generated from transient amplifying cells (arising from radial glia-like cells) migrate through the rostral migratory stream (RMS) to reach the olfactory bulb (Lois, C. et al. 1996). Here, immature neurons move toward glomeruli and differentiate into subtypes of interneurons (Lledo, P.M. et al. 2006). In the SGZ, intermediate progenitors are generated from proliferating radial and nonradial (Type I and Type II) precursor cells (Figure 1.3B). These

intermediate progenitors give rise to neuroblasts which are fate-determined to become neurons (Figure 1.3B) (Zhao, C. et al. 2006). Maturation of neuroblasts results in the development of immature granule cells and ultimately, fully functioning mature granule cells that have incorporated into the hippocampal circuitry (Figure 1.3B) (Zhao, C. et al. 2006; Ge, S. et al. 2008). Present day studies are beginning to unravel the significance of incorporating new neurons into existing brain circuitry and potential functional implications of adult neurogenesis for cognition, psychiatric diseases, and traumatic brain injuries (Zhao, C. et al. 2008; Ming, G.L. et al. 2011). Interestingly, adult neurogenesis can be regulated by many different intrinsic factors such as neurotrophins, cytokins, hormones, growth factors, neurotransmitters as well as by extrinsic factors and physiological stimuli (Zhao, C. et al. 2008; Ming, G.L. et al. 2011).

The sensitivity of the DG to fluctuations in neuronal birth and death may provide insight to the significance of neurogenesis. To elaborate, many studies have correlated changes in neurogenesis and cell survival in the DG to the regulation of depressive-like behavior (Dranovsky, A. et al. 2006; Sahay, A. et al. 2007; Balu, D.T. et al. 2009; Duman, R.S. 2009; Hanson, N.D. et al. 2011; Kubera, M. et al. 2011; Petrik, D. et al. 2012). To support the regulation of mood through neurogenesis, many positive factors that act as natural antidepressants such as exercise, environmental enrichment, and social interaction increase adult neurogenesis (van Praag, H. et al. 1999; Olson, A.K. et al. 2006; Veena, J. et al. 2009; Madronal, N. et al. 2010; Schloesser, R.J. et al. 2010). Some antidepressant drugs also enhance neurogenesis, and the timing of drug efficacy parallels the time it takes for new neurons to fully mature (Malberg, J.E. et al. 2000; Duman, R.S. et al. 2001; Malberg, J.E. et al. 2005; Boldrini, M. et al. 2009; Pechnick, R.N. et al. 2011). These antidepressants not only stimulate proliferation of neural progenitors, but also promote the maturation and synaptic plasticity of newly born granule neurons (Wang, J.W. et al. 2008). In mice exposed to chronic stress, neurogenesis is required for the beneficial effects of environmental enrichment that

reverse depressive-like behavior (Schloesser, R.J. et al. 2010). Additionally, the loss of neurogenesis through irradiation results in a loss of antidepressant efficacy, suggesting a dependency of antidepressant action on neurogenesis in the DG (Santarelli, L. et al. 2003; Wang, J.W. et al. 2008; Zhu, X.H. et al. 2010; Surget, A. et al. 2011). Ablation of neurogenesis also leads to dysfunction of the HPA axis, which regulates the stress response (Snyder, J.S. et al. 2011).

Upon stress, signaling molecules in the MAPK pathway that enhance neurogenesis such as pERK, pCREB, and BDNF are reduced in the hippocampus, and in some cases specifically the DG, only to be increased with antidepressant treatment (Qi, X. et al. 2006; Xu, Y. et al. 2006; Bland, S.T. et al. 2007; Qi, X. et al. 2008; Iio, W. et al. 2011). Also, the administration of proneurogenic factors like BDNF has an antidepressant effect, while blockade of BDNF in the DG results in a loss of anti-depressant efficacy (Shirayama, Y. et al. 2002; Adachi, M. et al. 2008; Schmidt, H.D. et al. 2010). In one elaborate study, intermittent hypobaric hypoxia (IH) was found to induce antidepressant-like effects in multiple behavioral tests of depression, and these antidepressant responses correlated with increased BDNF and DG neurogenesis (Zhu, X.H. et al. 2010). Interestingly, pharmacological and biological inhibition of BDNF signaling blocked both IH-induced elevations in neurogenesis and antidepressant responses (Zhu, X.H. et al. 2010). It is highly possible that signaling molecules in the MAPK pathway, such as BDNF, regulate mood through their positive effects on neurogenesis. In fact, ghrelin has been shown to activate ERK in several cell types containing endogenous or transfected *Ghsr* expression (Baldanzi, G. et al. 2002; Dixit, V.D. et al. 2004; Nanzer, A.M. et al. 2004; Mousseaux, D. et al. 2006), and ghrelin's proliferative and cell protective effects have been attributed to signaling of the MAPK pathway (Baldanzi, G. et al. 2002; Nanzer, A.M. et al. 2004). Therefore, ghrelin may exert its antidepressant effects through hippocampal GHSR signaling of the MAPK pathway, which in turn elevates BDNF and thereby promotes neurogenesis. Of course, it is also

conceivable that the antidepressant mechanism of BDNF is through a signaling pathway independent from, but parallel to, pathways that enhance neurogenesis. Thus, the utilization of a compound that promotes neurogenesis without targeting BDNF signaling would help distinguish the role of neurogenesis in antidepressant behavior.

On the other hand, negative regulators of neurogenesis, including chronic stress, drugs of abuse, old age, and social isolation, are often concurrent with shifts in mood and problems with learning or memory, implying alterations in hippocampal functioning potentially through reductions in neurogenesis (Gould, E. et al. 1997; Gould, E. et al. 1998; Yamaguchi, M. et al. 2004; McDonald, H.Y. et al. 2005; Bland, S.T. et al. 2006; Stranahan, A.M. et al. 2006; Arguello, A.A. et al. 2009; Yun, J. et al. 2010; Van Bokhoven, P. et al. 2011; Kubesova, A. et al. 2012). Importantly, the assortment of neurogenesis ablation studies have yielded inconsistent results on the effect of neurogenesis on mood, although these discrepancies are likely due to differences in methodology such as technique for ablation, behavioral test(s) performed, stress level of animals, and timing of study (Santarelli, L. et al. 2003; Holick, K.A. et al. 2008; Surget, A. et al. 2008; Bessa, J.M. et al. 2009; David, D.J. et al. 2009; Lagace, D.C. et al. 2010; Zhu, X.H. et al. 2010; Petrik, D. et al. 2012). Therefore, the loss of neurogenesis alone may not always precipitate depression, but in combination with particular stimuli, such as psychosocial stress, or with certain genetic variations, reductions in neurogenesis may lead to an increased susceptibility for more severe depressive-like behavior.

Overall, the strong associations found between neurogenesis and mood regulation support the idea that variations in neurogenesis may be instigating changes in depressive-like behavior. Furthermore, the strong ties of ghrelin to stress-associated changes in mood and reward preference, ghrelin's capacity to be neuroproliferative and neuroprotective, and reported interactions of ghrelin with the DG suggest that hippocampal changes in neurogenesis may

mediate the coordinated ghrelin behavioral response to psychosocial stress. In the following chapter of my thesis, Chapter 2, I will investigate this relationship between DG neurogenesis, ghrelin, and mood in depth.

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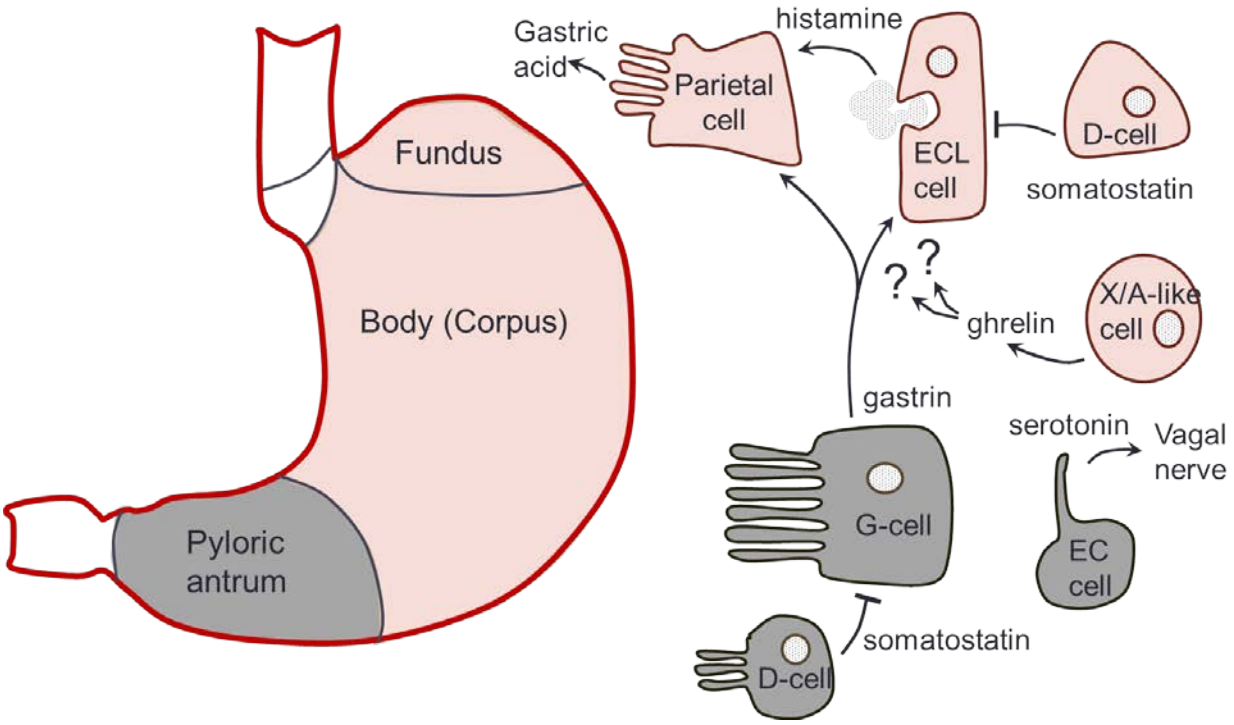

Figure 1.1. Gastric enteroendocrine cell types and function in gastric acid secretion. Left region of diagram represents the three main anatomical regions of the stomach. Right region of diagram represents major enteroendocrine cells working together to regulate gastric acid secretion. Arrows represent excitation through signaling while lines with a flat edge represent inhibition. The ECL cell releases histamine to stimulate gastric acid secretion from parietal cells. ECL cells are inhibited by somatostatin from D-cells and activated by gastrin from G-cells; somatostatin can also inhibit gastrin release from G-cells. The effect of ghrelin on each of these cell populations to influence gastric acid secretion remains unknown. Pink colored cells represent cell populations located in the fundus and body of the stomach, while gray colored cells represent cell populations in the pyloric antrum.

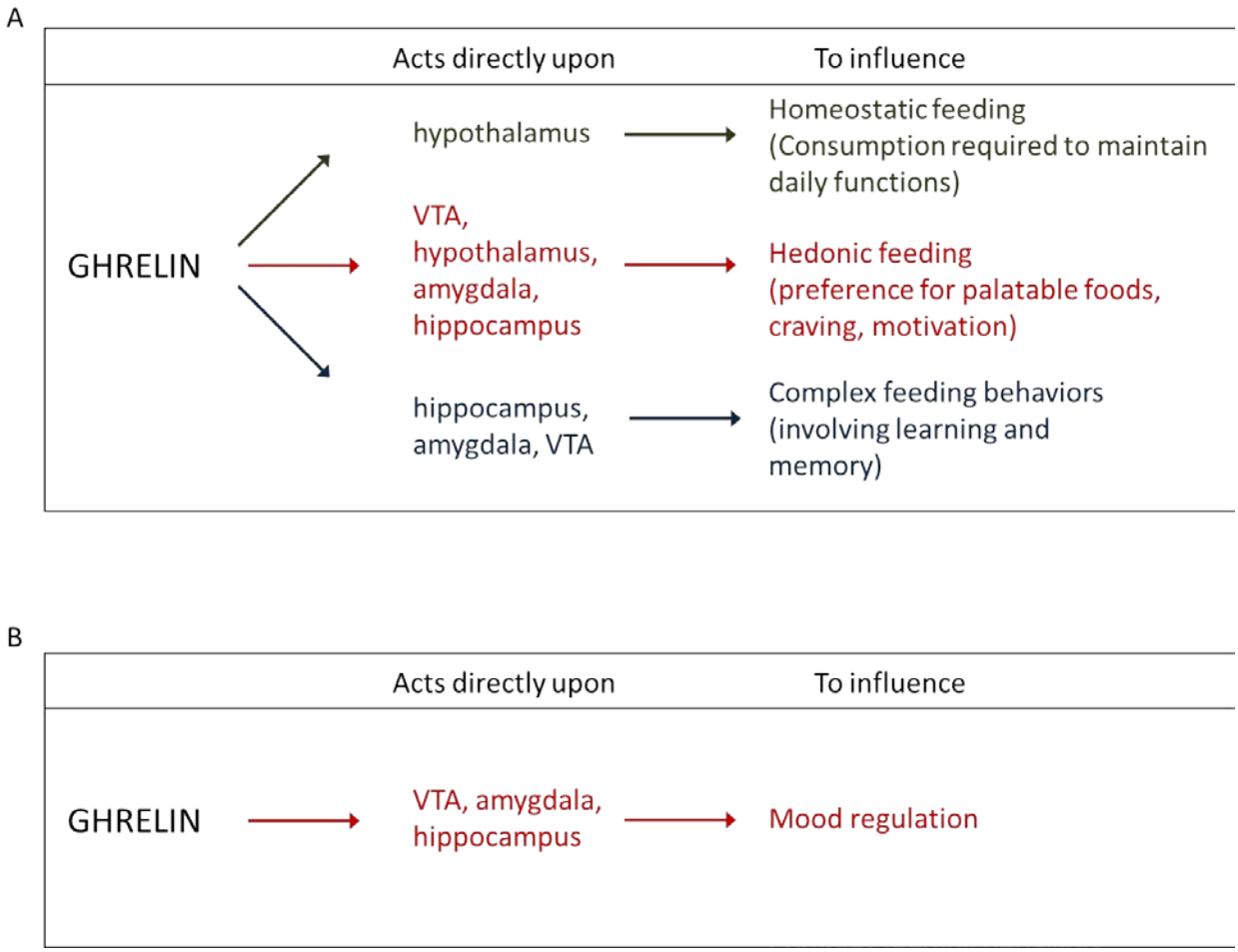

Figure 1.2. Central ghrelin action modulates feeding behavior and mood. This hypothetical model displays brain regions directly targeted by ghrelin to influence feeding behaviors (A) or mood regulation (B).

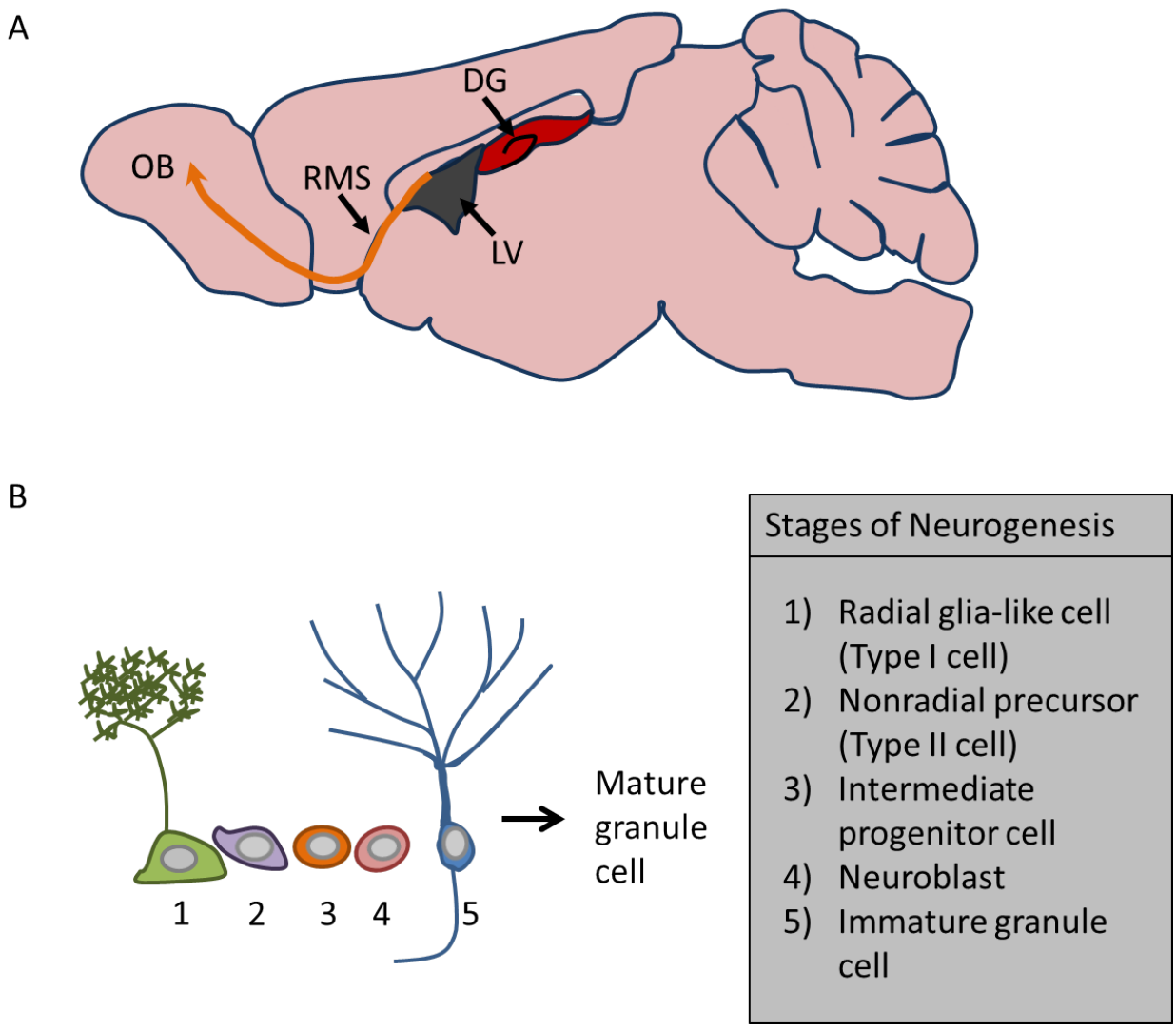

Figure 1.3. Process of neurogenesis in the adult brain. (A) Diagram representing the two brain regions highly characterized for maintaining neurogenesis throughout adulthood, the subgranular zone of the dentate gyrus (DG) and the subventricular zones of the lateral ventricles (LV). From the LV, neuroblasts migrate down the rostral migratory stream (RMS) to reach their destination, the olfactory bulb (OB), where they mature into neurons. (B) Representation of the stages a newborn DG cell passes through as it differentiates into a mature neuron (granule cell). (Ming and Song, 2011)

CHAPTER TWO

The P7C3-class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis

Adapted from: **Walker AK**, Rivera PD, Wang Q, Chuang J-C, Tran S, Osborne-Lawrence S, Estill SJ, Starwalt R, Huntington P, Morlock L, Naidoo J, Williams N, Ready JM, Eisch AJ, Pieper AA, Zigman JM. The P7C3-class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis. (2014). Molecular Psychiatry. *In press.*

ABSTRACT

Augmenting hippocampal neurogenesis represents a potential new strategy for treating depression. Here, this possibility was tested by comparing hippocampal neurogenesis in depression-prone ghrelin receptor (*Ghsr*)-null mice to that in wild-type littermates and by determining the antidepressant efficacy of the P7C3-class of neuroprotective compounds. Exposure of *Ghsr*-null mice to chronic social defeat stress (CSDS) elicits more severe depressive-like behavior than in CSDS-exposed wild-type littermates, and exposure of *Ghsr*-null mice to 60% caloric restriction fails to elicit antidepressant-like behavior. CSDS resulted in more severely reduced cell proliferation and survival in the ventral DG subgranular zone of *Ghsr*-null mice than in that of wild-type littermates. Also, caloric restriction increased apoptosis of DG subgranular zone cells in *Ghsr*-null mice, although it had the opposite effect in wild-type littermates. Systemic treatment with P7C3 during CSDS increased survival of proliferating DG cells, which ultimately developed into mature (NeuN+) neurons. Notably, P7C3 exerted a potent antidepressant-like effect in *Ghsr*-null mice exposed to either CSDS or caloric restriction,

while the more highly active analog P7C3-A20 also exerted an antidepressant-like effect in wild-type littermates. Focal ablation of hippocampal stem cells with radiation eliminated this antidepressant effect, further attributing the P7C3-class antidepressant effect to its neuroprotective properties and resultant augmentation of hippocampal neurogenesis. Finally, P7C3-A20 demonstrated greater proneurogenic efficacy than a wide spectrum of currently marketed antidepressant drugs. Taken together, these data confirm the role of aberrant hippocampal neurogenesis in the etiology of depression and suggest that the neuroprotective P7C3-compounds represent a novel strategy for treating patients with this disease.

INTRODUCTION

Despite the multitude of antidepressant drugs available to patients, major depression remains a significant cause of morbidity and mortality in our society. Thus, there is great need to further understand the mechanisms underlying depression in order to develop new treatments. To that end, investigating the relationship between the orexigenic gut hormone ghrelin and depression has unveiled interesting new links between this metabolic peptide and the regulation of mood. Ghrelin induces feeding by activating growth hormone secretagogue receptors (*Ghsr*; ghrelin receptor) in the hypothalamus, caudal brainstem, and elsewhere in the central nervous system (Kojima, M. et al. 1999; Nakazato, M. et al. 2001; Faulconbridge, L.F. et al. 2003; Horvath, T.L. et al. 2003). Within the hippocampus and ventral tegmental area, *Ghsr* mediates ghrelin's enhancement of reward-related behaviors (Jerlhag, E. et al. 2010; Chuang, J.C. et al. 2011; Perello, M. et al. 2012; Walker, A.K. et al. 2012), cue-potentiated feeding (Walker, A.K. et al. 2012; Kanoski, S.E. et al. 2013), hippocampal spine synaptic density and memory retention (Diano, S. et al. 2006). Ghrelin additionally confers neuroprotective efficacy in models of kainic acid hippocampal toxicity, spinal cord motor neuron excitotoxicity, dopaminergic neuron toxicity and oxygen glucose deprivation in hypothalamic and cortical

neurons (Chung, H. et al. 2007; Chung, H. et al. 2008; Andrews, Z.B. et al. 2009; Moon, M. et al. 2009; Lee, J. et al. 2010; Lee, J.Y. et al. 2010; Lim, E. et al. 2011). Notably, ghrelin also exerts antidepressant efficacy in rodent models of depression (Lutter, M. et al. 2008; Lutter, M. et al. 2009; Kluge, M. et al. 2011; Steiger, A. et al. 2011). In mice, raising ghrelin levels either directly through acute injection or indirectly via caloric restriction elicits an antidepressant response in the forced swim test (FST), a common screening tool for new candidate antidepressants(Lutter, M. et al. 2008). Additionally, elevated plasma ghrelin levels occur in mice exposed to CSDS, a model of prolonged psychosocial stress featuring aspects of major depression and posttraumatic stress disorder, and *Ghsr*-null mice exhibit more severe depressive-like behavior after CSDS than wild-type littermates (Lutter, M. et al. 2008; Chuang, J.C. et al. 2011; Golden, S.A. et al. 2011). Besides exaggerated depressive-like behavior, CSDS-exposed GHSR-deficient mice lack the hyperphagia and conditioned place preference for high fat diet otherwise present in CSDS-exposed wild-type mice (Lutter, M. et al. 2008; Chuang, J.C. et al. 2011; Patterson, Z.R. et al. 2013). Ghrelin is also elevated in other models of acute and chronic stress in animals, as well as in a model of psychosocial stress in people (Asakawa, A. et al. 2001; Kristenssson, E. et al. 2006; Rouach, V. et al. 2007; Ochi, M. et al. 2008; Patterson, Z.R. et al. 2010; Raspopow, K. et al. 2010; Patterson, Z.R. et al. 2013). Furthermore, *Ghsr*-polymorphism has been associated with major depression in humans, and administration of ghrelin improves mood in some patients with major depression (Nakashima, K. et al. 2008; Kluge, M. et al. 2011). Thus, stress-associated activation of ghrelin signaling may help protect against depression, while aberrant ghrelin signaling may confer increased sensitivity to stress-induced depression as well as changes to the usual metabolic and food reward behavioral responses to stress.

The mechanism by which ghrelin confers antidepressant efficacy has previously eluded the field, although clues in the literature point to involvement of hippocampus neurogenesis. For

example, changes in hippocampal neurogenesis and cell survival in the dentate gyrus (DG) have been correlated with depressive-like behavior (Kubera, M. et al. 2011; Snyder, J.S. et al. 2011; Eisch, A.J. et al. 2012; Petrik, D. et al. 2012), and antidepressants and environmental factors that elevate mood, such as exercise, environmental enrichment, and social interaction, increase the net magnitude of hippocampal neurogenesis (van Praag, H. et al. 1999; Malberg, J.E. et al. 2000; Olson, A.K. et al. 2006; Van Bokhoven, P. et al. 2011). By contrast, negative regulators of neurogenesis and cell survival, such as chronic stress, old age, drugs of abuse and social isolation, are associated with depressed mood (Eisch, A.J. et al. 2006; Stranahan, A.M. et al. 2006; Lutter, M. et al. 2008; Van Bokhoven, P. et al. 2011). Additionally, ablation of neurogenesis decreases the efficacy of some antidepressant drugs in rodents (Santarelli, L. et al. 2003; Sahay, A. et al. 2007; Surget, A. et al. 2008). It has also been reported that ghrelin potently stimulates hippocampal neurogenesis within the DG (Johansson, I. et al. 2008; Moon, M. et al. 2009; Li, E. et al. 2013; Li, E. et al. 2013). The antidepressant efficacy of compounds initially categorized as neuroprotective and/or proneurogenic, however, has not yet been described. In considering the convergence of evidence linking neurogenesis with depressive-like behavior, as well as the role of ghrelin in hippocampal neurogenesis and hippocampal distribution of *Ghsr* (Zigman, J.M. et al. 2006), it was hypothesized that the antidepressant efficacy of ghrelin might relate to its proneurogenic effect. If so, both depression-prone *Ghsr*-null mice and even wild-type mice with intact ghrelin signaling might be protected from stress-induced depression by pharmacologically augmenting hippocampal neurogenesis. Here, these hypotheses have been tested by utilizing the P7C3-series of neuroprotective compounds and *Ghsr*-null mice.

MATERIALS AND METHODS

For full material and methods, refer to supplementary materials and methods.

Animals

Male *Ghsr*-null and wild-type littermates on pure C57/BL/6J genetic background were generated and housed as described previously (Zigman, J.M. et al. 2006; Chuang, J.C. et al. 2011). All procedures were performed according to protocols approved by The University of Texas Southwestern Medical Center Institutional Animal Care and Use of Committee guidelines.

P7C3 compounds

P7C3 was from Asinex (Moscow, Russia). P7C3-A20 was prepared as described (Pieper, A.A. et al. 2010).

Behavioral testing

Behavioral tests were performed as described previously (Lutter, M. et al. 2008; Chuang, J.C. et al. 2011). P7C3 compounds were administered intraperitoneally at 9 a.m. and 5 p.m. daily, and BrdU was administered at 9 a.m. Body weights were unaffected by compounds (Fig. 2.12).

Chronic social defeat stress (CSDS)

CSDS was performed as previously described (Chuang, J.C. et al. 2011).

Hippocampus-directed cranial irradiation

Refer to online methods.

Caloric restriction

60% calorie restriction protocol was performed as previously described (Lutter, M. et al. 2008). Mice were provided with 60% of their usual daily calories, resulting in an 18-20% body

weight loss in both *Ghsr*-null and wild-type littermates (Fig. 2.12), as observed previously (Lutter, M. et al. 2008). Mice received twice-daily injections of either P7C3 (20 mg/kg/d in divided doses) or vehicle 5 days prior to and during 10 days of calorie restriction or ad-lib feeding. On Day 16, the forced swim test was performed for each mouse, as done previously (Lutter, M. et al. 2008).

Immunohistochemistry and stereology

Immunohistochemistry and quantification were performed as described previously (Malberg, J.E. et al. 2000; Pieper, A.A. et al. 2010; Chuang, J.C. et al. 2011).

Quantitative RT-PCR

Brain punch collection and quantitative PCR was performed as previously described (Chuang, J.C. et al. 2011; Walker, A.K. et al. 2012).

P7C3 brain penetration study

Refer to supplementary methods.

Comparison of P7C3 compounds and antidepressant drugs

Proneurogenic efficacies of P7C3 and P7C3-A20 were compared to vehicle (artificial cerebrospinal fluid) and several antidepressants (Sigma-Aldrich, St. Louis, MO), according to established methods (Pieper, A.A. et al. 2010; MacMillan, K.S. et al. 2011; De Jesus-Cortes, H. et al. 2012).

Statistical Analyses

GraphPad Prism 5.0 was used for statistical analysis. Significance was defined as $P < 0.05$. Data are presented as mean ± s.e.m.

RESULTS

Chronic stress severely reduces DG cell proliferation and survival in *Ghsr*-null mice.

The CSDS model of chronic psychosocial stress and major depression exposes male mice to repeated bouts of social subordination by an older and larger aggressor mouse for 5 min per day over 10 days (Berton, O. et al. 2006; Lutter, M. et al. 2008; Chuang, J.C. et al. 2011; Golden, S.A. et al. 2011). Afterwards, the stressed mouse typically shows significantly reduced social interaction with a novel mouse, reflected by decreased time spent in the interaction zone with a novel mouse and/or increased time spent in the corners of the testing chamber, mimicking stress-induced social avoidance and depression in humans (Berton, O. et al. 2006; Chuang, J.C. et al. 2011; Golden, S.A. et al. 2011). CSDS is a valuable model of depression because the social avoidance behavioral manifestation of depressive-like behavior is long-lasting and responsive to chronic, but not acute, antidepressant administration (Berton, O. et al. 2006; Krishnan, V. et al. 2007). Exposure of male *Ghsr*-null mice to CSDS elicited significantly more time in the corners and less time interacting with a novel mouse, compared to wild-type littermates (Fig. 2.1), consistent with the lab's previous reports (Lutter, M. et al. 2008; Chuang, J.C. et al. 2011).

To determine whether the more severe depressive phenotype in *Ghsr*-null mice correlated with effects on hippocampal neurogenesis, brain tissue was harvested from both CSDS-exposed and non-CSDS-exposed mice and performed immunohistochemistry for Ki67, a marker of proliferating cells, in the DG subgranular zone (SGZ) where newborn neural precursor cells proliferate. The dorsal 2/3 (septal and intermediate regions) and ventral 1/3 (temporal region) of the hippocampus was separately analyzed based on reported differences between these regions in neuronal projections, connectivity and gene expression profiles, as well as evidence differentially linking the ventral hippocampus to mood regulation and emotion-based learning and the dorsal hippocampus to spatial learning and memory (Hock, B.J., Jr. et al. 1998;

Moser, M.B. et al. 1998; Thompson, C.L. et al. 2008; Czerniawski, J. et al. 2009; Maggio, N. et al. 2009; Fanselow, M.S. et al. 2010; Tanti, A. et al. 2012). More specifically, adult neurogenesis in the ventral DG has been correlated with an antidepressant effect (Banasr, M. et al. 2006; Boldrini, M. et al. 2009; Kheirbek, M.A. et al. 2011). In addition, ghrelin delivery to the ventral hippocampus stimulates food intake, whereas delivery to the dorsal hippocampus does not (Kanoski, S.E. et al. 2013). Ventral hippocampus ghrelin administration also induces various motivational and learning-related eating behaviors (operant responding for sucrose reward and cue-potentiated feeding) (Kanoski, S.E. et al. 2013). Although both wild-type and *Ghsr*-null mice displayed an equally significant reduction in the number of Ki67+ cells in the dorsal DG following CSDS (Fig. 2.2a), there was significantly greater reduction in Ki67+ cells in the ventral DG of *Ghsr*-null mice than in wild-type littermates (Fig. 2.2b-f).

Because the vast majority of newborn hippocampal neural precursor cells in mice die by apoptosis, and pharmacologically inhibiting apoptosis augments hippocampal neurogenesis (Pieper, A.A. et al. 2010), the question arose whether apoptosis of newborn neural precursor cells might also be affected by CSDS. Therefore, adjacent tissue sections were immunohistochemically stained for activated caspase 3 (AC3), a marker of apoptotic cells. While CSDS had no effect on AC3+ cell numbers in the dorsal DG (Fig. 2.3a), the ventral DG of all animals displayed significantly more AC3+ cells after CSDS (Fig. 2.3b). Furthermore, significantly more AC3+ cell numbers were observed in the ventral DG of CSDS-exposed *Ghsr*-null mice than in CSDS-exposed wild-type littermates (Fig. 2.3b-l).

Taken together, these results demonstrate that the pool of proliferating cells contributing to hippocampal neurogenesis throughout the DG is decreased in both wild-type and *Ghsr*-null littermates after CSDS, with the negative effect on hippocampal neurogenesis further exacerbated in the ventral DG by virtue of additionally elevated rates of cell death. This net

reduction in ventral DG cell survival is significantly more pronounced in *Ghsr*-null mice and thus parallels the exacerbated depression-like behavior observed after CSDS in *Ghsr*-null mice as compared to wild-type littermates. Localization of this ghrelin effect to the ventral DG may be related to the known ventral DG role in regulating mood (Moser, M.B. et al. 1998; Czerniawski, J. et al. 2009; Maggio, N. et al. 2009; Tanti, A. et al. 2012) and to a higher baseline *Ghsr* expression within the ventral DG relative to the dorsal DG, as demonstrated here by quantitative RT-PCR of ventral and dorsal hippocampal tissue punches taken from wild-type mice (Fig. 2.4).

P7C3 compounds augment DG neurogenesis in chronic stress-exposed mice.

Next, it was investigated whether protecting neural precursor cells from CSDS-associated apoptosis might help protect against the depressive-like phenotype. To test this hypothesis, P7C3 was utilized, which is a neuroprotective aminopropyl carbazole that elevates hippocampal neurogenesis by blocking apoptosis of DG neural precursor cells without affecting the number of glial cells (Pieper, A.A. et al. 2010; MacMillan, K.S. et al. 2011; De Jesus-Cortes, H. et al. 2012). P7C3 and its active analogs have been shown to enhance hippocampal-dependent spatial learning in young rats that have undergone blunt-traumatic brain injury and in aged rats, and they also have been investigated for potential therapeutic effects in neurodegenerative diseases (Pieper, A.A. et al. 2010; De Jesus-Cortes, H. et al. 2012; Tesla, R. et al. 2012; Blaya, M.O. et al. 2013). The neuroprotective effect of P7C3 is thought to involve protection of mitochondrial membrane integrity, though the precise molecular target of this novel class of molecules has not yet been identified (Pieper, A.A. et al. 2010). A twice-daily schedule of intraperitoneal P7C3 injections (20 mg/kg/d in divided doses) was initiated two days prior to CSDS and continued throughout the 10 day procedure for both wild-type and *Ghsr*-null littermates. Throughout the 10 days of CSDS, the thymidine analog bromodeoxyuridine (BrdU; 50 mg/kg i.p.) was administered daily to label newborn cells (Fig. 2.5a). Liquid chromatography-

tandem mass spectrometry analysis of plasma and brain P7C3 concentrations revealed that similar P7C3 levels were achieved in wild-type and *Ghsr*-null mice (Fig. 2.6).

Similar to the above Ki67 results, immunohistochemical examination of brain tissue from these mice revealed that CSDS reduced the number of cells with BrdU incorporation (a marker of dividing cells and/or cell survival) in both vehicle-treated wild-type and *Ghsr*-null mice throughout the DG, with a more significant reduction in the ventral DG of *Ghsr*-null mice (Fig. 2.5b-c, f-i, l-q). In both wild type and *Ghsr*-null mice, P7C3 administration during CSDS blocked CSDS-induced reductions in BrdU+ cells throughout both dorsal and ventral regions of the DG (Fig. 2.5b-c, f-k). In parallel, vehicle-treated mice displayed an elevation of AC3+ cells only in the ventral DG after CSDS, an outcome associated with a stronger *P*-value in *Ghsr*-null mice (Fig. 2.5d-e) and blocked by P7C3 administration (Fig. 2.5e-k). Thus, treatment with P7C3 blocked the elevated programmed cell death usually observed in the DG after CSDS. Dual-label immunohistochemistry for both BrdU and NeuN, which is a marker of mature neurons, revealed that P7C3 administration during CSDS resulted in significantly more BrdU+/NeuN+ cells than vehicle administration, reflective of increased survival of proliferating DG neural precursor cells that develop into mature neurons (Fig. 2.7). This, combined with the observed decreases in DG subgranular zone AC3+ cells, supports a net effect of increased hippocampal neurogenesis in mice receiving P7C3 during CSDS exposure.

P7C3 compounds reduce depression in chronic stress-exposed mice.

Next, P7C3 and two P7C3 chemical analogs were employed to investigate whether P7C3-mediated preservation of ventral DG neurogenesis during CSDS might result in an antidepressant-like effect. The analog P7C3-A20 substitutes a fluoride at the hydroxyl position in the linker region, conferring significantly greater neuroprotective efficacy than P7C3(De Jesus-Cortes, H. et al. 2012), as demonstrated in animal models of Parkinson's disease (De

Jesus-Cortes, H. et al. 2012), amyotrophic lateral sclerosis (Tesla, R. et al. 2012) and traumatic brain injury(Blaya, M.O. et al. 2013). Conversely, the analog P7C3-S184 replaces bromines on the carbazole moiety with chlorines, and the aniline moiety with a naphthyl amine, leaving the analog devoid of neuroprotective activity (De Jesus-Cortes, H. et al. 2012). In CSDS-exposed wild-type mice, P7C3 treatment had no significant effect on depression-like behavior (Fig. 2.8 a,c). However in CSDS-exposed *Ghsr*-null mice, both P7C3 and P7C3-A20 showed antidepressant efficacy, reflected by reduced time spent in the corners of the testing chamber and increased time spent in the interaction zone (Fig. 2.8b,d). The more highly active analog P7C3-A20 also showed antidepressant efficacy in CSDS-exposed wild-type mice, as reflected by significantly reduced time spent in the corners of the testing chamber (Fig. 2.8a). The inactive analog P7C3-S184 had no effect on stress-induced depressive-like behavior in either genotype (Fig. 2.8a-d). Neither P7C3 nor the two tested analogs influenced social interaction test performance in non-CSDS-exposed control mice (Fig. 2.9). Thus, P7C3 displayed antidepressant efficacy in the stressed, depression-prone *Ghsr*-null mice, while the more highly active analog P7C3-A20 exhibited antidepressant efficacy both in stressed *Ghsr*-null mice and in their more resilient, stressed wild-type littermates. Lack of an antidepressant effect of P7C3-S184, which lacks neuroprotective activity, supports the notion that the antidepressant efficacy of P7C3 and P7C3-A20 is due to neuroprotection.

The antidepressant efficacy of P7C3-A20 was also tested using the CSDS protocol in mice that received focal irradiation targeting the hippocampus as compared to non-irradiated sham controls. Hippocampus-directed cranial irradiation was performed at a dose of 15 gray, which ablates proliferating cells without causing significant mRNA elevations in inflammatory markers at 1 month post-irradiation (Ko, H.G. et al. 2009; Moravan, M.J. et al. 2011). Administration of P7C3-A20 to irradiated *Ghsr*-null and wild-type mice during CSDS resulted in significantly more time spent in the corners and less time in the interaction zone as compared to

that observed in P7C3-A20-administered sham mice (Fig. 2.8e,f). Therefore, cranial irradiation-induced ablation of proliferating DG cells resulted in a loss of P7C3-A20's antidepressant efficacy, further suggesting that the antidepressant efficacy of P7C3 and P7C3-A20 is due to their ability to augment the net magnitude of hippocampal neurogenesis by blocking death of proliferating neural precursor cells.

P7C3 blocks apoptosis and restores the antidepressant response to caloric restriction in *Ghsr*-null mice.

Prolonged caloric restriction in wild-type mice elicits an antidepressant-like response in the FST (decreased immobility), whereas this effect is not observed in *Ghsr*-null mice (Lutter, M. et al. 2008). After confirming this finding (Lutter, M. et al. 2008) (Fig. 2.10d-e), its relationship to differences in DG cell survival was investigated, similar to the immunohistochemical studies performed following CSDS. *Ghsr*-null and wild-type littermates were exposed to 60% caloric restriction for 10 days, vs. ad libitum food access to control mice. Five days prior to and throughout the 10-days of restricted feeding, mice received either P7C3 or vehicle. On Day 16, FST performance was assessed, followed by quantification of AC3+ cells in the DG (Fig. 2.10a). Surprisingly, the number of AC3+ cells after caloric restriction was reduced throughout the DG in wild-type mice, yet increased in *Ghsr*-null mice (Fig. 2.10b-c, f-p). Whereas P7C3-treatment did not further reduce the number of AC3+ cells in caloric-restricted wild-type mice (Fig. 2.10b-c), it did block the increase in apoptosis observed after caloric restriction in *Ghsr*-null mice (Fig. 2.10b-c). Notably, this effect correlated with significantly decreased immobility of *Ghsr*-null mice in the FST, with no behavioral effect in wild-type littermates (Fig. 2.10d-e). Thus, the neuroprotective efficacy of P7C3 in *Ghsr*-null mice restores the antidepressant-like effect of caloric restriction to that normally observed in wild-type mice (Fig. 2.10d-e).

P7C3-A20 has greater proneurogenic efficacy than current antidepressants.

Upon confirming a role for P7C3-related compounds in the regulation of depressive-like behavior, the proneurogenic efficacy of P7C3 and P7C3-A20 was then compared to several currently marketed antidepressants, using screening conditions identical to those by which P7C3 was discovered (Pieper, A.A. et al. 2010). Continuous and direct intracerebroventricular infusion of P7C3 or P7C3-A20 over a one-week period of time into the left lateral ventricle markedly augmented BrdU labeling in the contralateral hemisphere by about 100% or 160%, respectively (Fig. 2.11). By contrast, only four out of fourteen antidepressants significantly increased DG BrdU labeling over vehicle. In particular, the norepinephrine-dopamine reuptake inhibitor bupropion, the monoamine oxidase inhibitor phenelzine, and the tricyclic antidepressants clomipramine and desipramine increased BrdU labeling by about 49%, 52%, 75% and 102%, respectively. Of those, only clomipramine and desipramine were statistically as effective as P7C3 in elevating BrdU+ cell number, and none was statistically as effective as P7C3-A20. The remaining antidepressants tested, including the selective serotonin reuptake inhibitors paroxetine, citalopram, fluoxetine and sertraline, the serotonin-norepinephrine reuptake inhibitor venlafaxine, the noradrenergic and specific serotonergic antidepressant mirtazapine, the monoamine oxidase inhibitor tranylcypromine, and the tricyclic antidepressants nortriptyline and imipramine, did not affect BrdU labeling. Thus, the proneurogenic efficacy of the P7C3-class of compounds is superior to a wide spectrum of antidepressants representing the major classes currently prescribed. If indeed, as the current studies suggest, augmentation of hippocampal neurogenesis is crucial for the manifestation of antidepressant efficacy of endogenously generated substances (i.e. ghrelin) or exogenously administered pharmacologic agents, then the magnitude of proneurogenic efficacy offered by highly active members of the P7C3 class suggests that this chemical scaffold may serve as a basis for developing a new and improved class of antidepressants.

Addendum to publication:

P7C3 has no effect on anxiety-like behaviors.

Extended studies of P7C3's potential therapeutic benefits involved determining its influence on behaviors related to anxiety. In order to elevate endogenous ghrelin levels, *Ghsr*-null mice and wild-type littermates were placed under the 60% calorie restriction protocol or ad-libitum feeding, as performed previously (Lutter, M. et al. 2008). Using this paradigm, the Zigman lab has previously shown that caloric restriction induces ghrelin-mediated anxiolytic behavior in the EPM test (Lutter, M. et al. 2008). In the current study, mice received twice-daily injections of either P7C3 (20 mg/kg/d in divided doses) or vehicle 5 days prior to and during 10 days of calorie restriction or ad-lib feeding. Following the final day of caloric restriction, relative levels of anxiety were assessed using the open field (OF) test and the elevated plus maze (EPM) test.

P7C3 administration had no impact on time spent in the center or periphery in the OF test and did not affect time spent in the open or closed arms in the EPM tests (Fig. 2.13, Fig. 2.14). Hence, anxiety-related behaviors are not altered with P7C3 administration, and thus far, its behavioral efficacy seems to target depressive-like behavior specifically. Locomotor activity also was not influenced by P7C3 administration since the recorded movement distances during testing did not differ between study groups (Fig. 2.13, Fig. 2.14). Strikingly, in both *Ghsr*-null and wild-type mice, caloric restriction reduced time spent in the center and increased time spent in the periphery in the OF test (Fig. 2.13). These results suggest that caloric restriction prompts anxiogenic behavior in the OF test through a mechanism independent of ghrelin activity. Moreover, my study did not reveal the previously observed anxiolytic effect of caloric restriction in the EPM, suggesting that the stress of daily injections may have modified mouse behavior.

Notably, conflicting studies of ghrelin's influence on anxiety-like behavior reveal the complexity of the relationship, as differing methodologies divulge contradicting results. Particularly, variations in the behavioral tests used, assorted transgenic/knockout mouse models, diverse methods of ghrelin induction, and systemic versus local manipulations of ghrelin signaling have created a controversy as to how ghrelin signaling affects anxiety (Kristensson, E. et al. 2007; Carvajal, P. et al. 2009; Chuang, J.C. et al. 2010; Currie, P.J. et al. 2012; Kajbaf, F. et al. 2012; Spencer, S.J. et al. 2012; Currie, P.J. et al. 2014). Initial studies revealed that ghrelin administration intracerebroventricularly (ICV) in rats increased latency time in the step down inhibitory avoidance test, increasing freezing behavior, and decreased the number of entries into open spaces and time spent in the open arms in the OF and EPM tests, respectively (Carlini, V.P. et al. 2002). These findings suggest an anxiogenic effect of ghrelin action. A follow-up study found that direct administration of ghrelin into the hippocampus, amygdala, or dorsal raphe nucleus dose-dependently produced anxiogenesis, as determined by the EPM and step-down test inhibitory avoidance test (Carlini, V.P. et al. 2004). Direct administration of ghrelin into hypothalamic regions elicited anxiety-like behavior, although only at doses four times the amount required to produce an orexigenic response (Currie, P.J. et al. 2012). The high dosage of ghrelin required for this particular anxiogenic response may lead one to question the physiological relevance of this finding. Chronic infusion of ICV ghrelin administration for 4 weeks also yielded an increase in anxiety-like behavior in rats (Hansson, C. et al. 2011). In one example of a non-mammalian species, central ghrelin to chicks increased anxiety-like behavior in the OF test (Carvajal, P. et al. 2009).

Alternatively, it was observed that peripheral ghrelin administration or caloric restriction produced anxiolytic effects in the EPM, but lack of ghrelin signaling in *Ghsr*-null mice inhibited this anixiolytic response (Lutter, M. et al. 2008). Ghrelin knockout mice elicited greater anxiety-like behavior after acute restraint stress, and Spencer et al. suggests that ghrelin reduces

anxiety after stress by stimulation of the hypothalamic-pituitary-adrenal axis (Spencer, S.J. et al. 2012). Additionally, the high anxiety Wistar Kyoto rat strain was found to have lower fasting levels of circulating ghrelin as compared to Sprague-Dawley rats correlating low ghrelin signaling with higher anxiety-like behavior (Kristensson, E. et al. 2007). Interestingly, direct administration of ghrelin into the ventrolateral and ventromedial subnuclei of the lateral amygdala produced anxiolytic responses in the open field and elevated plus maze under the condition that rats were not allowed access to food after ghrelin administration (Alvarez-Crespo, M. et al. 2012). If rats were allowed to feed after ghrelin administration, there was no effect on anxiety behavior (Alvarez-Crespo, M. et al. 2012). Conversely, direct injection of ghrelin into the BLA nuclei of the amygdala induced an anxiogenic response, and pretreatment with the peptide urocortin-1 potentiated this response (Currie, P.J. et al. 2014). These studies suggest that even within the amygdala, ghrelin signaling produces differential effects based on which specific nuclei it interacts with.

Organization of future studies based on whether ghrelin is manipulated as opposed to the ghrelin receptor will likely provide some clarity. In addition, elevating ghrelin through pharmacological means may result in supraphysiological levels of ghrelin, while methods to induce endogenous elevations, although more natural, may also elevate other molecular signals that confound results. Therefore, in order to elucidate the rapport between ghrelin signaling and anxiety, prolonged research is required, with careful attention to experimental and procedural approach.

The localization of ghrelin receptor expression in DG cell populations

Although it has been established that ghrelin receptors are expressed in the DG, and ghrelin enhances DG neuroproliferation in the DG and ghrelin receptors are expressed are expressed in the DG (Zigman, J.M. et al. 2006; Moon, M. et al. 2009), it is not known in what

DG cell type ghrelin receptors are expressed. The expression of ghrelin receptors in mature granule neurons may indirectly influence neurogenesis, while expression of ghrelin receptors in cells during certain stages of proliferation would have a direct impact on neurogenesis. In order to fully understand the manner in which ghrelin is modulating the process of neurogenesis, from proliferation to maturation, it is important to determine whether ghrelin is capable of acting directly on specific populations of proliferating cells.

The *Ghsr*-eGFP mouse, in which the *Ghsr* promotor controls eGFP expression, was used to visualize *Ghsr*-expressing cells in the hippocampus (Smith, J.T. et al. 2013). Hippocampal brain sections from these mice were immunohistochemically labeled for doublecortin (DCX), which is widely used as a marker for neurogenesis, representing neural precursor cells and immature neurons (Couillard-Despres, S. et al. 2005; Karl, C. et al. 2005). Neuronal precursor cells express DCX for 2-3 weeks after initial expression, which occurs while the cell is actively dividing (Brown, J.P. et al. 2003). Once cells begin to express NeuN at around 2 weeks post birth, downregulation of DCX occurs, signaling the stage in which neuronal maturation occurs (Brown, J.P. et al. 2003). This marker was chosen because its expression specifies neural precursors rather than all proliferating cells, which could include glia.

My preliminary data reveal an absence of eGFP fluorescence in the SGZ of the DG, which is the zone in which proliferating neuronal precursors are typically located (Fig. 2.15, Fig. 2.16). Expression of eGFP fluorescence was most intense in the granule cell layer (GCL), suggesting ghrelin receptors may be expressed in the later stages of proliferation or during differentiation into mature neurons (Fig. 2.15, Fig. 2.16). It is also likely that ghrelin receptors are expressed in embryonically-generated granule neurons, given the widespread eGFP expression in the GCL. Furthermore, there was a lack of significant colocalization between eGFP fluorescence and DCX immunolabeling, suggesting that ghrelin does not directly act upon

this population of neural precursors (Fig. 2.15). Otherwise, it is possible that eGFP expression intereferes with DCX-immunolabeling, presenting a potential caveat of this particular method.

Attempting to more thoroughly characterize the DG cell that also expresses ghrelin receptors, I performed qPCR on FAC-sorted DG cells from *Ghsr*-eGFP mice. The eGFP-enriched population of cells (eGFP+) had a significant augmentation of *Ghsr* expression, confirming that ghrelin receptors are expressed at higher levels in cells that also express eGFP (Fig. 2.17a). Relative expression of *Emx1* was also enriched, which is a gene that displays expression restricted to the hippocampus and cerebral cortex in the adult brain (Fig. 2.17b) (Guo, H. et al. 2000). Emx1 has also been implicated in the regulation of adult hippocampal neurogenesis and is involved in embryonic neural development of the dorsal telencephalon (Yoshida, M. et al. 1997; Hong, S.M. et al. 2007). Therefore, a population of DG cells that express both *Emx1* and *Ghsr* may exist in the DG, and continuing studies are necessary to reveal the importance of ghrelin action on these cells and exactly how it influences neurogenesis.

Furthermore, I developed preliminary data determining levels of *Prox1* expression in eGFP+ cells as compared to eGFP- cells. Prox1 is a protein highly expressed in the DG, and it has been associated with intermeditate progenitor cell maintenance and granule cell maturation (Lavado, A. et al. 2010). My assessment of this gene was based on the hypothesis that ghrelin receptors are expressed in cells at a later stage of neurogenesis, since Prox1 is involved in the maturation of adult-generated granule cells. Although my preliminary data did not reveal a significant enrichment of *Prox1* expression in eGFP+ cells, I believe that increasing the low sample number (N=3) may yield more reliable results (Fig. 2.17c). If *Prox1* truly is not expressed in *Ghsr*-expressing DG cells, it is possible that *Ghsr* expression is highly specific to a

particular DG cell type. Therefore, a central theme of future ghrelin studies should be to uncover the functional significance of DG cells coexpressing *Ghsr* and *Emx1*.

DISCUSSION

In the current study, impaired hippocampal neurogenesis has been identified as a general contributing factor to the depression associated with chronic psychosocial stress in these mouse models. Furthermore, effects seen in *Ghsr*-null mice suggest that hippocampal neuroprotection is a primary mechanism by which stress-induced elevations in ghrelin protect against what would otherwise be worsened stress-associated depression. These new insights led the way to determining the antidepressant efficacy of the P7C3 class of neuroprotective compounds in these animal models of depression. Indeed, P7C3 blocked both the decrease in hippocampal neurogenesis and the exacerbated depression-like behavior observed in CSDS-exposed *Ghsr*-null mice. Notably, even depression-like behavior observed in CSDS-exposed wild-type mice was minimized by P7C3-A20, a more highly active analog of P7C3. Absence of a behavioral response to P7C3-S184, a P7C3 analog that lacks neuroprotective efficacy, further supports specificity of the neuroprotective properties of P7C3 and P7C3-A20 in conferring antidepressant efficacy. Furthermore, the lack of these behavioral effects in mice exposed to hippocampus-directed cranial irradiation even more strongly links neuroprotection of P7C3 compounds to the observed antidepressant response.

Interestingly, in the current study, P7C3 administration did not significantly increase the number of BrdU+ cells in the DG of non-stressed control animals, a finding that differs from the original manuscript reporting the discovery of P7C3 (Pieper, A.A. et al. 2010) (Fig 2.5a-d). This difference may be attributed to changes in experimental technique. In particular, the original screen by which P7C3 was discovered utilized singly-housed 12-week-old adult male mice

completely deprived of environmental enrichment. This design was employed to maintain basal neurogenesis at a consistently low level for purposes of the discovery screen (Pieper, A.A. et al. 2010). By contrast, the current group of non-stressed control animals consisted of younger (8-weeks-of-age) mice that were group housed (in the same cage with a member of the same strain across a perforated divider) under conditions of normal environmental enrichment. Both younger age and social activity with group housing are associated with higher rates of baseline hippocampal neurogenesis (Olson, A.K. et al. 2006; Stranahan, A.M. et al. 2006), and thus may have masked the effect of P7C3 -- the least active member of the P7C3-class of neuroprotective chemicals – in the non-stressed control mice. Nonetheless, here, P7C3 was observed to potently augment neurogenesis in pathological conditions associated with elevated cell death of newborn hippocampal neural precursor cells.

Effects of ghrelin and P7C3 on hippocampal neurogenesis and depression-like behavior and a mechanistic link between the two were observed not only in mice exposed to CSDS, but also in mice exposed to 10-day caloric restriction, which also elevates circulating ghrelin. Similar to what was observed in CSDS-exposed *Ghsr*-null mice, the inability of *Ghsr*-null mice to mount the usual antidepressant response to caloric restriction was associated with acquired deficits in DG cell survival, as both the increase in apoptotic DG cells and the depression-like FST behavior observed in *Ghsr*-null animals were normalized by treatment with P7C3.

That said, the differential changes in hippocampal neurogenesis induced by the 10-day 60% caloric restriction protocol in *Ghsr*-null mice vs. wild type littermates were slightly different than those induced by CSDS. In particular, the number of AC3+ cells after caloric restriction was reduced throughout the DG in wild type mice, yet increased in *Ghsr*-null mice. CSDS, though, increased AC3+ hippocampal cell numbers in the ventral DG of both wild-type and *Ghsr*-null mice, with significantly more AC3+ cells observed in *Ghsr*-null mice as compared to

wild-type littermates. The different degrees of CSDS-induced social avoidance observed in *Ghsr*-null and wild type littermates suggest a dosage effect of defective hippocampal neurogenesis in driving depression-like behavior. However, if indeed hippocampal neurogenesis alone drives antidepressant-like responses, then an unchanged number of AC3+ DG cells (instead of an increased number) might have been expected in the calorically-restricted *Ghsr*-null mice, which showed similar immobility times compared to *ad lib*-fed *Ghsr*-null mice. P7C3 nonetheless was able to prevent the caloric restriction-induced elevation in AC3+ DG cell numbers in *Ghsr*-null mice, which is believed to have resulted in the restoration of the usual antidepressant-like behavioral response to the caloric restriction protocol.

Altogether, the more severe depressive-like behavior and exaggerated decrease in hippocampal neurogenesis observed in stress-exposed *Ghsr*-null mice, the depressive-like behavior and increased hippocampal cell death in caloric-restricted *Ghsr*-null mice, and the prevention of those effects by administration of active P7C3 compounds suggest a key protective role of ghrelin's inherent proneurogenic capacity in mediating mood responses to chronic stress and moderate caloric restriction. In addition, these findings further demonstrate the impact of DG neurogenesis on regulation of depressive-like behavior, with protection against exacerbated neurogenic loss conferring beneficial effects for depressive-behavior.

It is also important to mention that whereas some of the antidepressant agents tested here previously had been shown to enhance neurogenesis, the same was not observed in this assay. This lack of neurogenic efficacy potentially may be accounted for by differences in animal species, route of administration, dose, and administration duration. Regarding administration duration, previous studies demonstrated increased hippocampal BrdU labeling in response to fluoxetine after 14 days (Malberg, J.E. et al. 2000; Wang, Y. et al. 2011) and 28 days of administration but not after 1 day or 5 days (Malberg, J.E. et al. 2000). Also, enhanced

adult hippocampal neurogenesis was observed after 14 days of paroxetine and 21 days of tranylcypromine (Malberg, J.E. et al. 2000; Qiu, G. et al. 2007). While the effects of P7C3 and P7C3-A20 administration on neurogenesis has not been examined after 2 weeks of administration, the rapidity in which these P7C3 compounds affected neurogenesis in addition to the magnitude of their effect could contribute to their potential superiority over current antidepressant agents.

Overall, these results suggest that individuals with depression associated with insufficient ghrelin or ghrelin resistance might be particularly responsive to treatment with neuroprotective agents, as embodied by the P7C3 class. For instance, individuals who have undergone Roux-en-Y gastric bypass weight loss surgery have a higher rate of suicide than the general population (Adams, T.D. et al. 2007). As some studies have demonstrated decreased plasma ghrelin levels following Roux-en-Y gastric bypass (Thaler, J.P. et al. 2009), neuroprotection by the P7C3 class of compounds may help protect from depression by counteracting the impact of aberrant ghrelin signaling on hippocampal neurogenesis. Future studies are needed to assess the role of impaired hippocampal neurogenesis as a contributory factor to other forms of depression besides those associated with chronic psychosocial stress or defective ghrelin signaling. Also, while it was demonstrated that blocking loss of hippocampal neurons by administering P7C3 compounds during CSDS protects against the usual depressive-like behavioral response, future studies will be needed to determine whether the P7C3 compounds also will diminish CSDS-induced depression when administered after CSDS. The current study provides hope that the chemical scaffold represented by P7C3 and P7C3-A20 will provide a basis for optimizing and advancing a new class of antidepressants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY METHODS

Animals and housing

Male *Ghsr*-null and wild-type littermates on a pure C57/BL/6J genetic background were generated as described previously (Zigman, J.M. et al. 2006; Chuang, J.C. et al. 2011). Mice were housed in a 12-hour-light/dark cycle with ad libitum access to water and regular chow (Teklad Global Diet 16% Protein Diet [2016]; Harlan Teklad, Madison, WI) unless otherwise stated. All procedures were performed according to protocols approved by The University of Texas Southwestern Medical Center Institutional Animal Care and Use of Committee guidelines.

P7C3 compounds

P7C3 was from Asinex (Moscow, Russia) and P7C3-A20 was prepared as described (Pieper et al., 2010).

Behavioral testing

Behavioral tests were performed as described previously (Lutter, M. et al. 2008; Chuang, J.C. et al. 2011). Injections of P7C3 compounds were administered at 9 a.m. and 5 p.m. daily, and BrdU was administered at 9 a.m., as per the schedule in the main text and Figures 3a and 5a. All mice were weighed each morning to calculate doses. Body weights were unaffected by administration of the compounds (Fig. 2.12).

Chronic social defeat stress (CSDS)

CSDS was performed similar to methods used previously in the lab (Chuang, J.C. et al. 2011). Test mice were housed individually in large cages paired with a CD1 aggressor mouse, separated by a plastic divider with holes so as to allow sensory contact. For 5 min a day, the plastic divider was removed, exposing test mice to the CD1 mouse. After 5 min of exposure to the CD1 mouse, test mice were again separated and paired with a new CD1 mouse for the remainder of the 24 hours. This process was repeated for 10 days. Control mice were housed in equivalent cages with members of the same strain and handled daily. The social interaction test consists of placing mice in an arena with a small cage at one end. The movements of test mice were tracked for 2.5 min in the absence of another mouse, followed by 2.5 min in the presence of a novel CD1 mouse, or the “target”. Ethovision 3.0 software (Noldus, Leesburg, Virginia) measured the duration spent in the interaction zone or in the corners. As done previously, data are presented as time spent in the interaction zone when the target is present or time spent in the corners when the target is present (Chuang, J.C. et al. 2011).

Focal cranial irradiation

Ionizing radiation was delivered to 8-12 week old mice via the X-RAD 225Cx self-contained irradiation system (Precision X-Ray). It has a custom collimator that delivers a 6 x 14

X-ray beam at a rate of 4 Gy/min (225 kV, 13 mA) for 3.44 min to achieve a cumulative dose of 15 Gy. All irradiated mice were anesthetized with isoflurane (1.5-2.5%) before positioning in the irradiator with the skull located directly under the collimator for cranial irradiation. Mice then received a single exposure of 15 Gy. For sham controls, the mice received isoflurane but no radiation. All animals were monitored post-procedurally for any ill effects and returned to normal housing after being observed to be fully alert and responsive. Four weeks after irradiation or sham treatment, the CSDS protocol was performed on these mice followed by the social interaction test.

Caloric restriction study

The 60% calorie restriction protocol was performed as previously (Lutter, M. et al. 2008). This protocol, in which mice are provided daily with 60% of their usual daily calories, results in an 18-20% body weight loss in both *Ghsr*-null and wild-type littermates (Fig. 2.12), as observed previously (Lutter, M. et al. 2008). Mice received twice-daily injections of either P7C3 (20 mg/kg/d in divided doses) or vehicle 5 days prior to and during 10 days of calorie restriction or ad-lib feeding, replicating the injection protocol used for the CSDS studies. On Day 16, the forced swim test was performed for each mouse as done previously (Lutter, M. et al. 2008).

Immunohistochemistry and stereology

Immunohistochemistry and quantification were performed as described previously (Malberg, J.E. et al. 2000; Ables, J.L. et al. 2010; Pieper, A.A. et al. 2010; Chuang, J.C. et al. 2011; Walker, A.K. et al. 2013). Mice first were anesthetized and perfused transcardially with formalin (or paraformaldehyde for Fig. 2.11). Brains were extracted, coronally sectioned into 8 equal series at 25 µm thickness (or 5 equal series at 40 µm thickness for Fig. 2.11) using a sliding microtome, and then mounted onto Superfrost slides (Fisher Scientific, Richardson, TX), using previously described methods (Zigman, J.M. et al. 2006; Chuang, J.C. et al. 2011).

Standard procedures for immunolabeling of slides were employed consisting of antigen retrieval, 1 hr in blocking solution, overnight incubation in primary antibody solution, and 1 hr in appropriate secondary antibody solution(s) (Malberg, J.E. et al. 2000; Pieper, A.A. et al. 2010; Walker, A.K. et al. 2013). Image editing software Adobe Photoshop CS2 (San Jose, CA) was used to adjust brightness and contrast of photomicrographs and prepare figures.

Ki67, AC3, and AC3/BrdU immunolabeling

Primary antibodies included Rabbit-anti-Ki67 (1:1000, Vector Labs, Burlingame,CA), rabbit-anti-AC3 (1:450, Cell Signaling, Beverly, MA), and mouse-anti-BrdU (1:100, Roche Diagnostic, Mannheim, Germany). For double immunolabeling of AC3 (rabbit-anti-AC3) and BrdU (mouse-anti-BrdU), primary antibodies were incubated simultaneously, and incubation in secondary antibodies occurred separately. Counting of labeled cells was done manually at a magnification of 40X on a Zeiss Axioskop 2 microscope (Carl Zeiss Inc., Thornwood, NY). For each brain, labeled cells were counted in all hippocampal sections from one of the 8 series (or one of the 5 series for Fig. 2.11). Final quantification of the whole DG of each mouse was obtained by adding labeled cell counts from all sections and multiplying by the number of series. Dorsal DG or ventral DG cell counts were obtained by adding labeled cell counts for either the septal and intermediate regions combined, which comprise the dorsal 2/3 of sections, or the temporal region which comprises the ventral 1/3 of sections (Moser, M.B. et al. 1998; Thompson, C.L. et al. 2008; Fanselow, M.S. et al. 2010). The respective totals were then multiplied by the number of series. Photomicrograph images were taken using Axiovision software.

BrdU/NeuN immunolabeling

For double labeling BrdU (rat-anti-BrdU, 1:400, Accurate Chemicals, Westbury, NY) and NeuN (mouse-anti-NeuN, 1:500, Millipore, Billerica, MA), the tissue was incubated with the

NeuN primary antibody overnight, followed by a fluorophore-conjugated secondary antibody. Full pretreatment for antigen unmasking was then performed, followed by the standard procedure for BrdU immunolabeling (Ables, J.L. et al. 2010).

For proportional analysis, colocalization of signals was determined by scanning and optical sectioning in the Z plane of double-immunofluorescence labeled sections with a Zeiss Axiovert 200/LSM510 confocal microscope (emission wavelengths 488, and 633). 60 – 110 BrdU+/NeuN+ cells per animal were sampled from 3 sections across the longitudinal axis of the hippocampus (-0.82 mm to -4.24 mm from from Bregma), n ≥ 4 per group) were analyzed. The total number of BrdU+/NeuN+ cells was calculated by multiplying the total BrdU+ counts by the proportion of BrdU+/NeuN+ cells.

Quantitative RT-PCR

Mice were euthanized by live decapitation, and brains were processed with a brain matrix and a 15-guage blunt needle to excise 1-mm thick tissue punches from various sites. RNA was extracted from tissues, processed and reverse-transcribed, and the resulting cDNA was used as template for quantitative PCR, as previously described (Chuang, J.C. et al. 2011; Walker, A.K. et al. 2012). Quantitative PCR was performed using iTaq SYBER Green Supermix with Rox (BioRad, Hercules, CA) and previously validated *Ghsr* and *cyclophilin* primer sets in an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), as described (Chuang, J.C. et al. 2011; Walker, A.K. et al. 2012). Relative mRNA levels were determined with *cyclophilin* used as the housekeeping gene and calculated with the comparative threshold cycle (ΔΔCt) method (Chuang, J.C. et al. 2011).

P7C3 brain penetration study

Mice were injected with either P7C3 (10 mg/kg) or vehicle twice every day at 9 am and 5 pm for a total of 19 days. Pharmacokinetic analysis was performed as done previously (Pieper, A.A. et al. 2010). Six hours after the last injection of either P7C3 or 0.9% saline (for vehicle treated mice), mice were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) for blood and brain collection. Blood was collected transcardially using an EDTA-coated syringe, and blood was dispensed into EDTA-coated vials on ice. Whole brain was then removed, weighed, and snap-frozen in liquid nitrogen. Blood was spun down to separate plasma, which was kept at -80°C until analysis was performed. Brain tissue was homogenized in a 3-fold volume of PBS to prepare lysates. Liquid chromatography and mass spectrometry were used to determine levels of P7C3.

Comparison of P7C3 compounds and antidepressant drugs

Proneurogenic efficacies in the DG of P7C3 and P7C3A20 were compared to those of vehicle (artificial cerebrospinal fluid) and several marketed antidepressant compounds (Sigma-Aldrich, St. Louis, MO) according to established methods (Pieper, A.A. et al. 2010; MacMillan, K.S. et al. 2011; De Jesus-Cortes, H. et al. 2012). In brief, mice were housed individually in cages without running wheels or any form of environmental enrichment, in order to create as low of a baseline level of hippocampal neurogenesis as possible. Compounds (10 μM, i.c.v.) were administered for 7 days via subcutaneously implanted Alzet osmotic minipumps connected to a cannula directed into the left lateral ventricle, with implantation day designated as day 0. Starting on day 1, mice also received daily injections of BrdU (50 mg/kg, i.p.) at 9 am daily, for 6 days. Twenty four hours after the sixth BrdU injection, mice were transcardially perfused as described above.

Statistical analyses

Two-way ANOVAs with Bonferonni post-hoc tests were performed when analyzing the effect of genotype and stress or genotype and injection/stress treatment on social interaction or cell counts for Ki67, AC3, and BrdU. One-way ANOVA with Dunnett's post-hoc test was performed when assessing the effect P7C3 analogs on social interaction, the effect of anti-depressants on BrdU cell number, or the effect of brain region on gene expression. If unequal variance was indicated by Bartlett's test, log transformation was performed prior to statistical analysis. Two-tailed unpaired t-tests were performed when analyzing P7C3 brain penetration data. Significant *p*-value was defined as $p < 0.05$. GraphPad Prism 5.0 was used for all statistical analysis. Data are presented as mean ± s.e.m.

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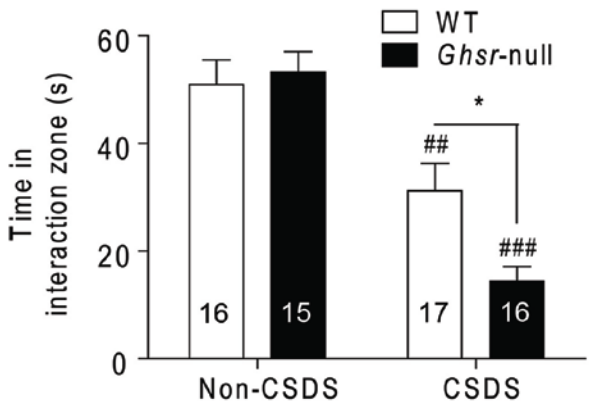

Figure 2.1. Depressive-like behavior in *Ghsr*-null and wild-type littermates.

Time spent in the social interaction zone when the target is present in CSDS-exposed and non-CSDS-exposed *Ghsr*-null and wild-type littermates. **P* < 0.05, comparing genotypes. ## *P* < 0.01, ### *P* < 0.001, as compared to non-CSDS controls.

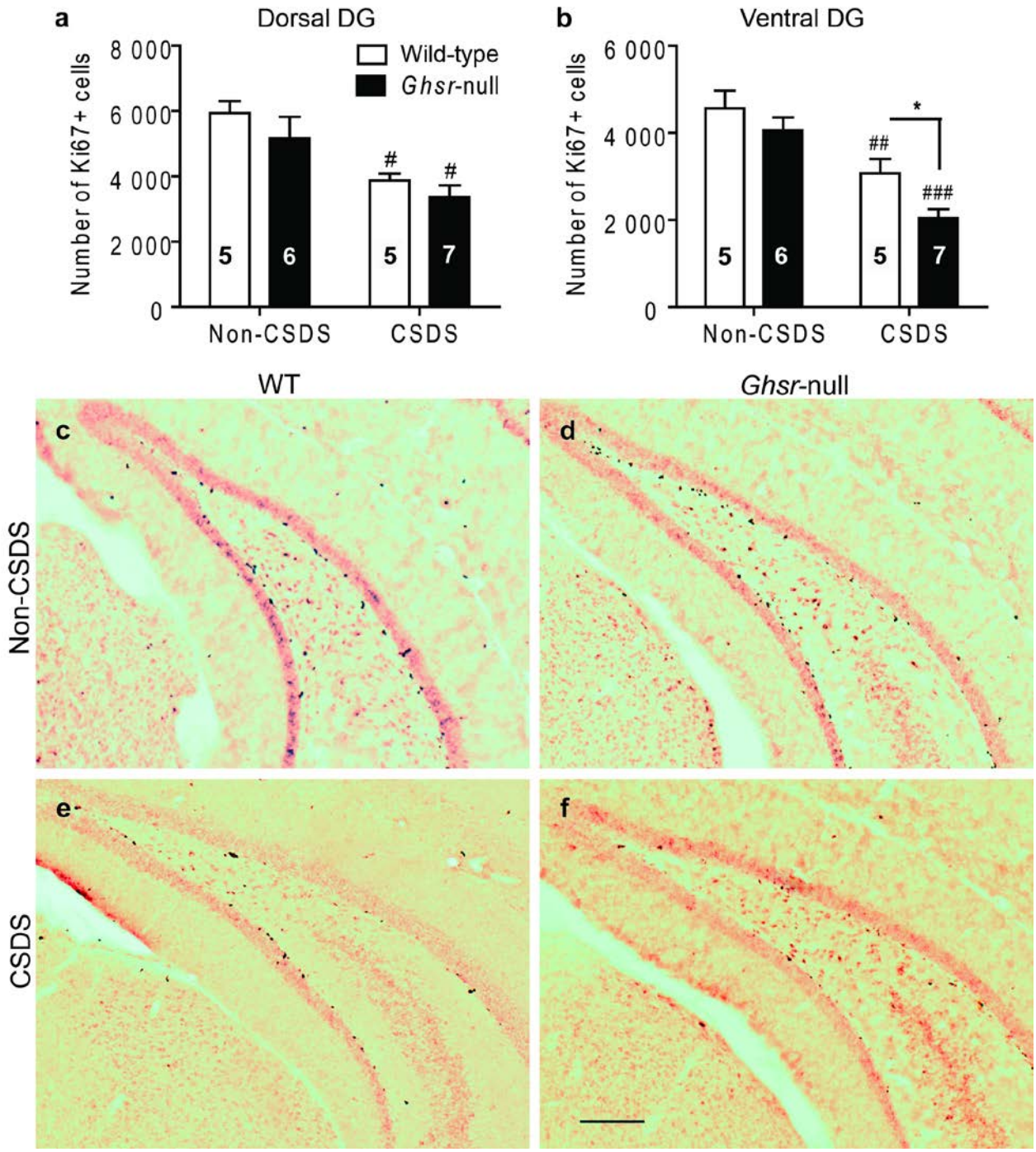

Figure 2.2. Effect of CSDS on cellular proliferation in the DG of *Ghsr*-null and wild-type littermates. (a,b) Ki67+ cell counts in the SGZ of the dorsal (a) and ventral (b) DG of CSDS-exposed or non-CSDS-exposed control mice. Group sizes (n) indicated within bars. (c-f) Representative photomicrograph images of Ki67-immunolabeled brain sections in the ventral

DG of CSDS-exposed and non-CSDS-exposed wild-type and *Ghsr*-null mice. Legend in (a) pertains to (b). Scale bar in (f; 300 µm) pertains to (c-f). $^{*}P < 0.05$, comparing genotypes. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, comparing treatment.

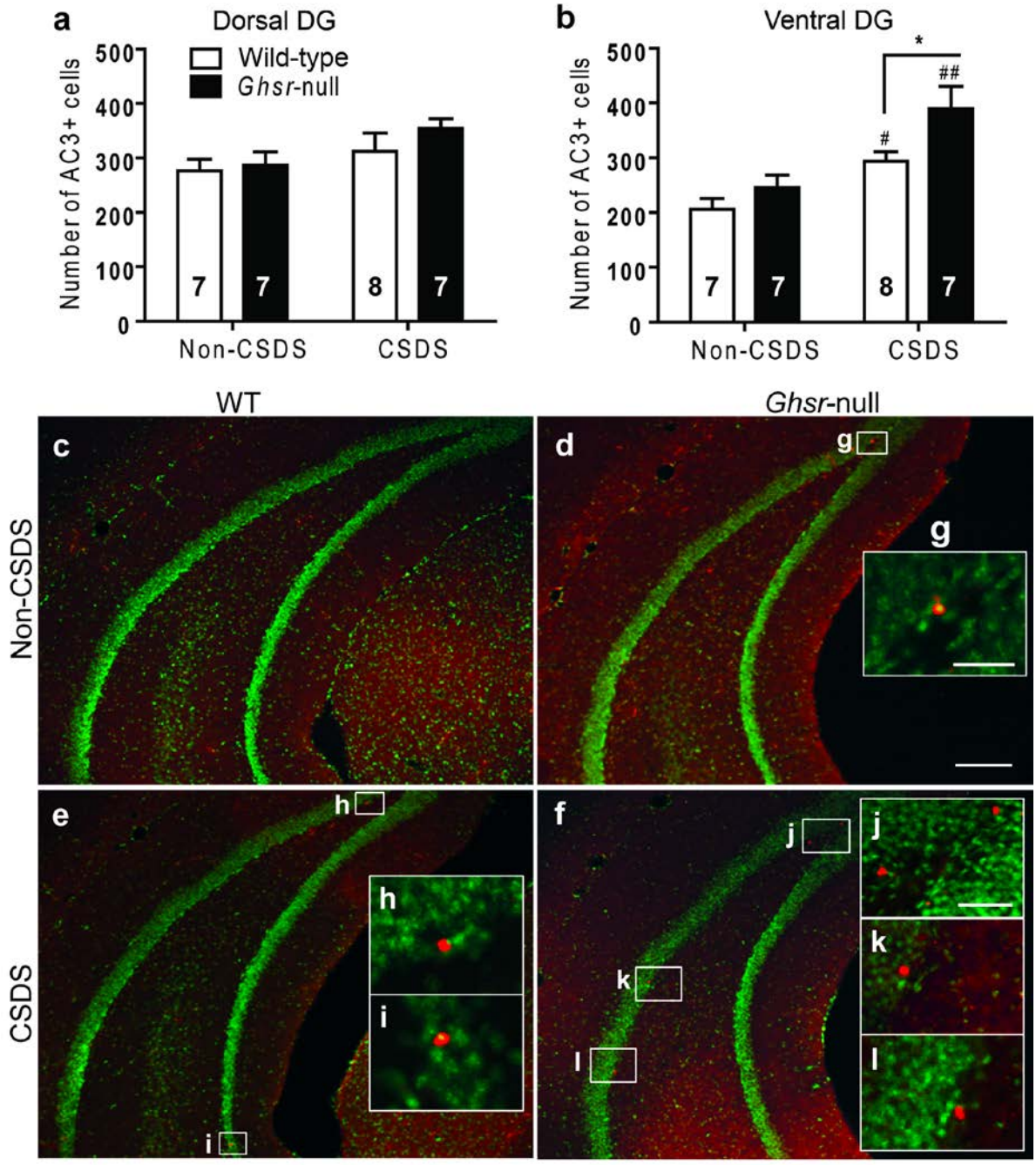

Figure 2.3. Effect of CSDS on apoptosis in the DG of *Ghsr*-null and wild-type littermates. (a,b). AC3+ cell counts in the SGZ of the dorsal (a) and ventral (b) DG of CSDS-exposed or non-CSDS-exposed mice. Group sizes indicated. (c-f) Representative photomicrograph images of the ventral DG from each study group. (g-l) Magnified images of AC3+ cells. Legend in (a) pertains to (b). Scale bar in (f; 300 µm) pertains to (c-f), in (g; 50 µm) pertains to (g-i), in (j; 75 µm) pertains to (j-l). $^{*}P < 0.05$, comparing genotypes. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, comparing treatment.

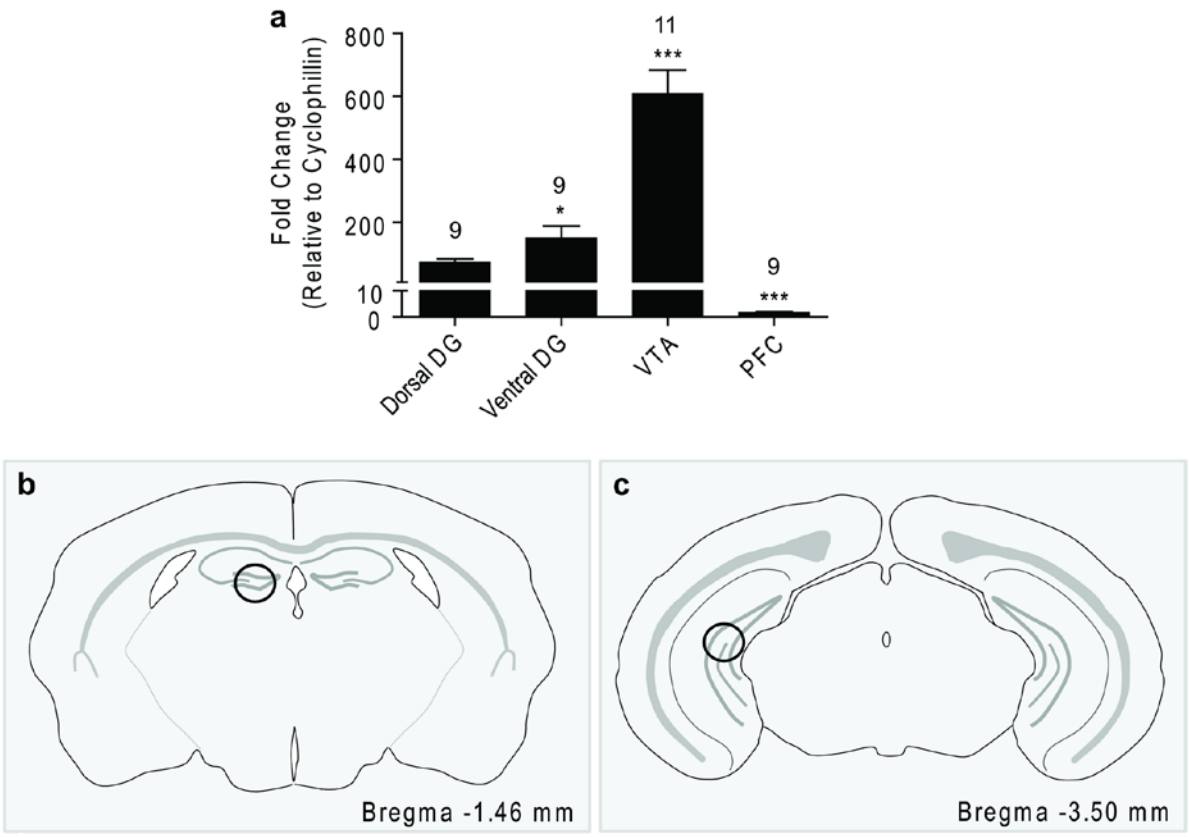

Figure 2.4. Relative *Ghsr* mRNA expression across brain regions in wild-type mice. (a) Relative *Ghsr* mRNA expression in the dorsal DG, ventral DG, ventral tegmental area (VTA), and prefrontal cortex (PFC). (b-c) Image traces of coronal sections representing dorsal (b) and ventral (c) regions where punches were collected for qPCR analysis. Group sizes indicated above bars. *$P < 0.05$, *** $P < 0.001$, as compared to the dorsal DG.

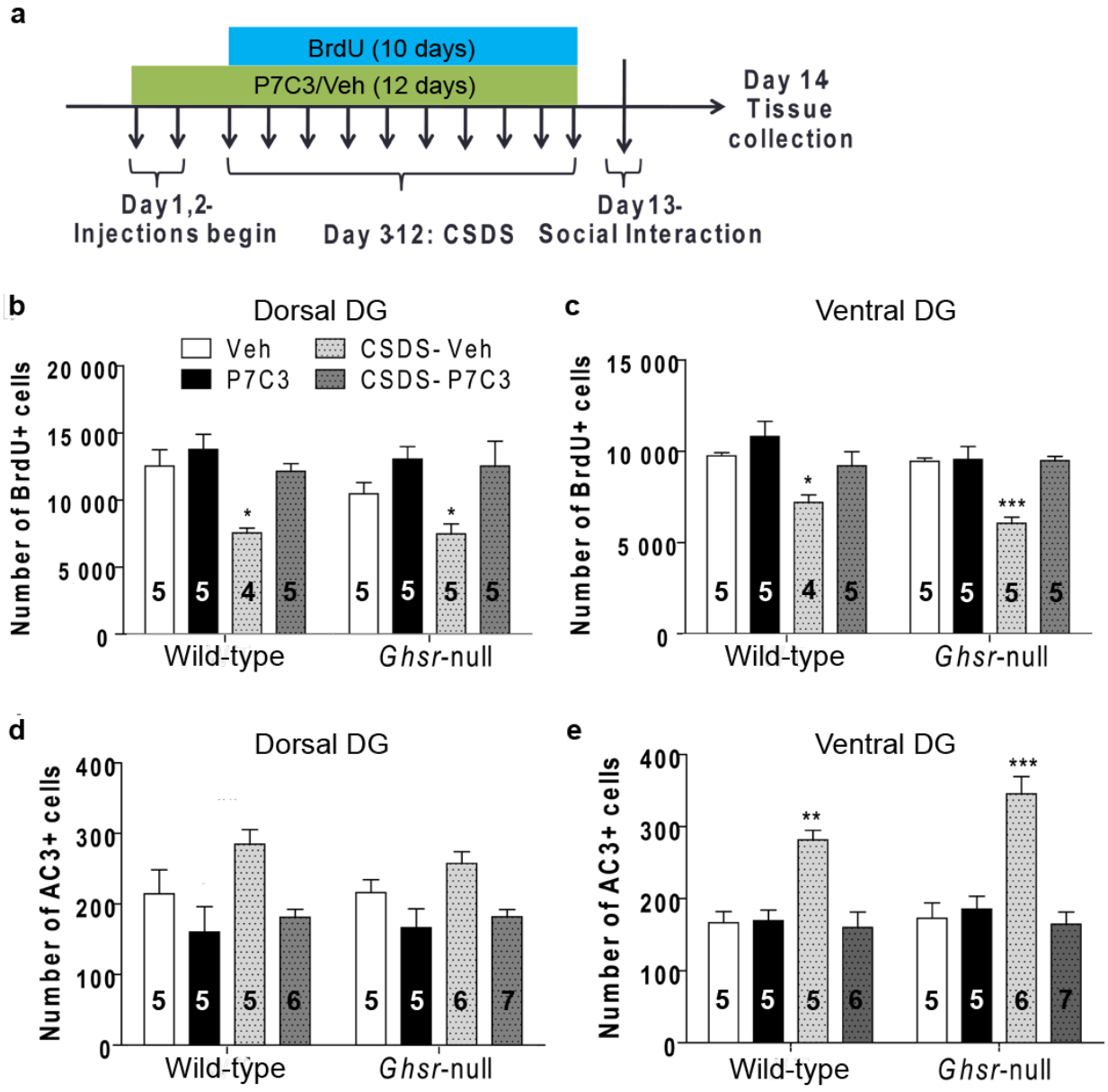

Figure 2.5a-e. Effects of P7C3 on DG cellular proliferation and apoptosis following CSDS. (a) Protocol schematic. (b-e) BrdU+ and AC3+ cell counts in the dorsal and ventral DG of CSDS-exposed and non-CSDS-exposed mice treated with P7C3 vs. vehicle. Group sizes indicated. *P < 0.05, ** P < 0.01, *** P < 0.001, as compared to respective non-CSDS-exposed, vehicle-treated control group.

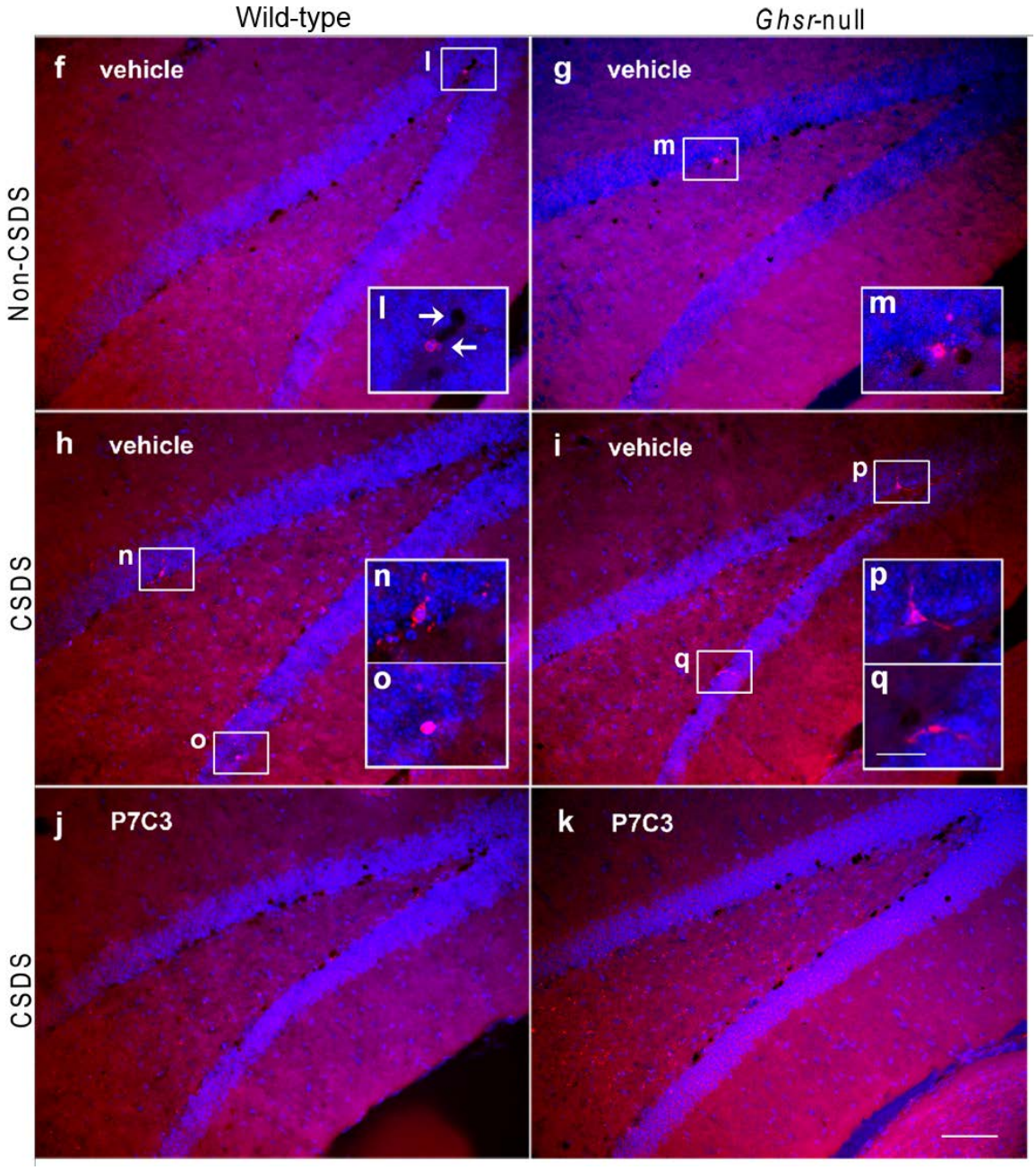

Figure 2.5f-q. Effects of P7C3 on DG cellular proliferation and apoptosis following CSDS. (f-k) Representative photomicrograph images of double-immunolabeled (BrdU and AC3) ventral DG sections. (l-q) Magnified images of immunoreactive cells (AC3+, leftward-facing arrow; BrdU+, rightward-facing arrow). Scale bar in (k; 100 µm) pertains to (f-k), in (q; 25 µm) pertains to (l-q).

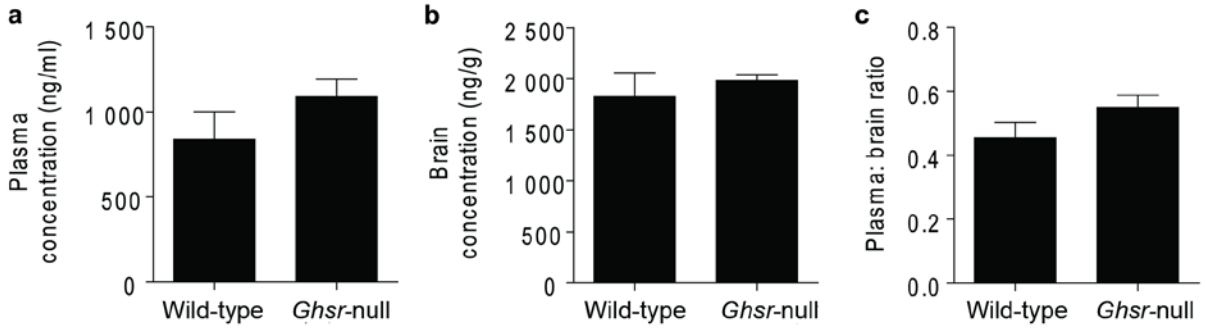

Figure 2.6. P7C3 brain penetration. (a-b) P7C3 levels measured in the plasma (a) or brain (b) of *Ghsr*-null mice and wild-type littermates 6 hrs after final P7C3 injection. (c) Plasma to brain ratio of P7C3 levels in *Ghsr*-null mice and wild-type littermates. (N=3/group)

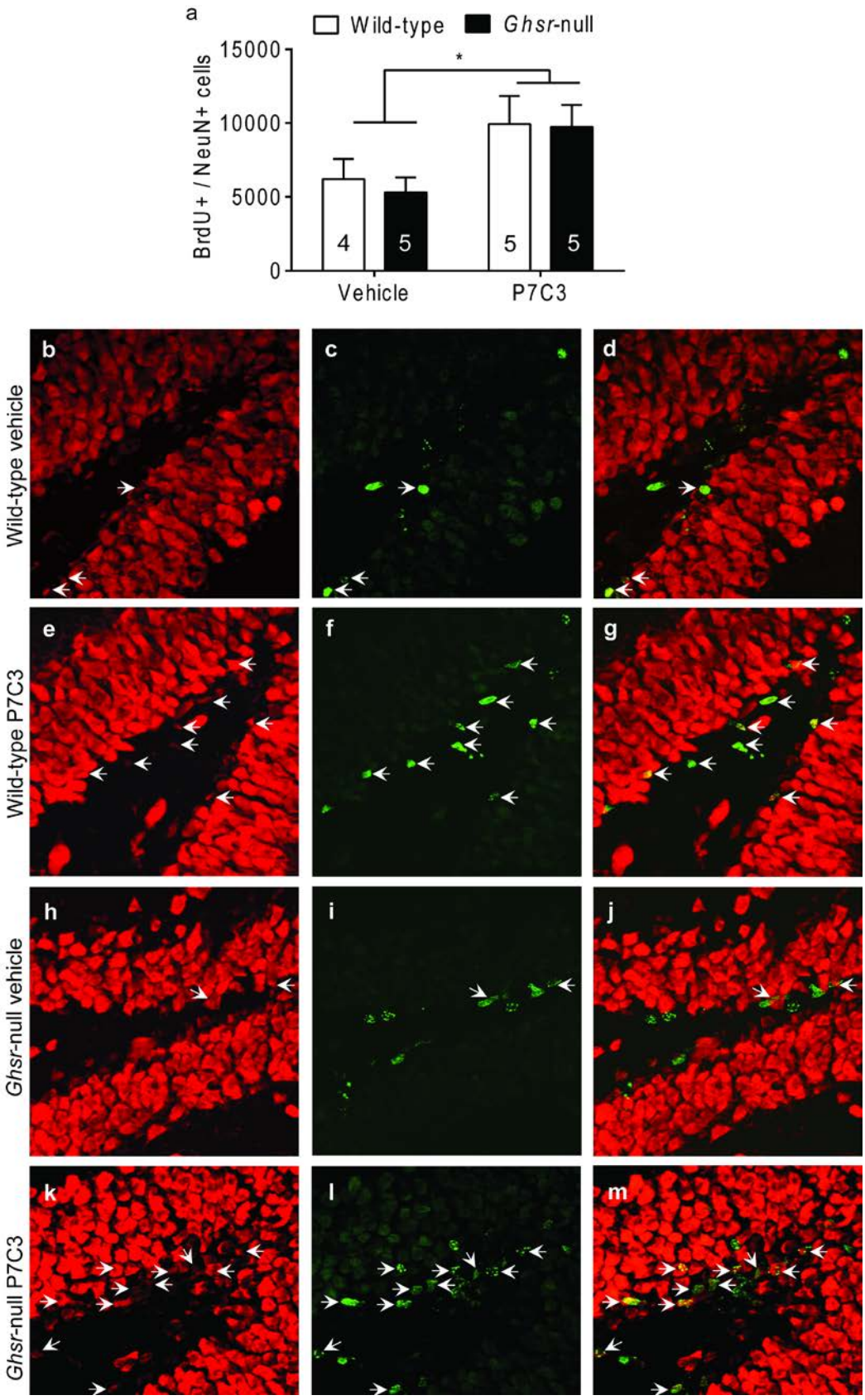
a
Wild-type
Ghsr-null
BrdU+ / NeuN+ cells
15000
10000
5000
0
*
4
5
5
5
Vehicle
P7C3
b
c
d
e
f
g
h
i
j
k
l
m
Wild-type vehicle
Wild-type P7C3
Ghsr-null vehicle
Ghsr-null P7C3

Figure 2.7. Effect of P7C3 administration on mature neuron development. (a) Number of BrdU+/NeuN+ cells in wild-type and *Ghsr*-null mice administered vehicle or P7C3. Group sizes as indicated. (b-m) Representative photomicrograph images of DG sections with NeuN+ cells shown in red (b,e,h,k), BrdU+ cells shown in green (c,f,i,l), and colocalization of BrdU+/NeuN+ cells (d,g,j,m). Representative BrdU+/NeuN+ cells are distinguished by arrows. Scale bar in (e; 20 μm) pertains to (b-e). $*P < 0.05$ effect of compound by two-way ANOVA.

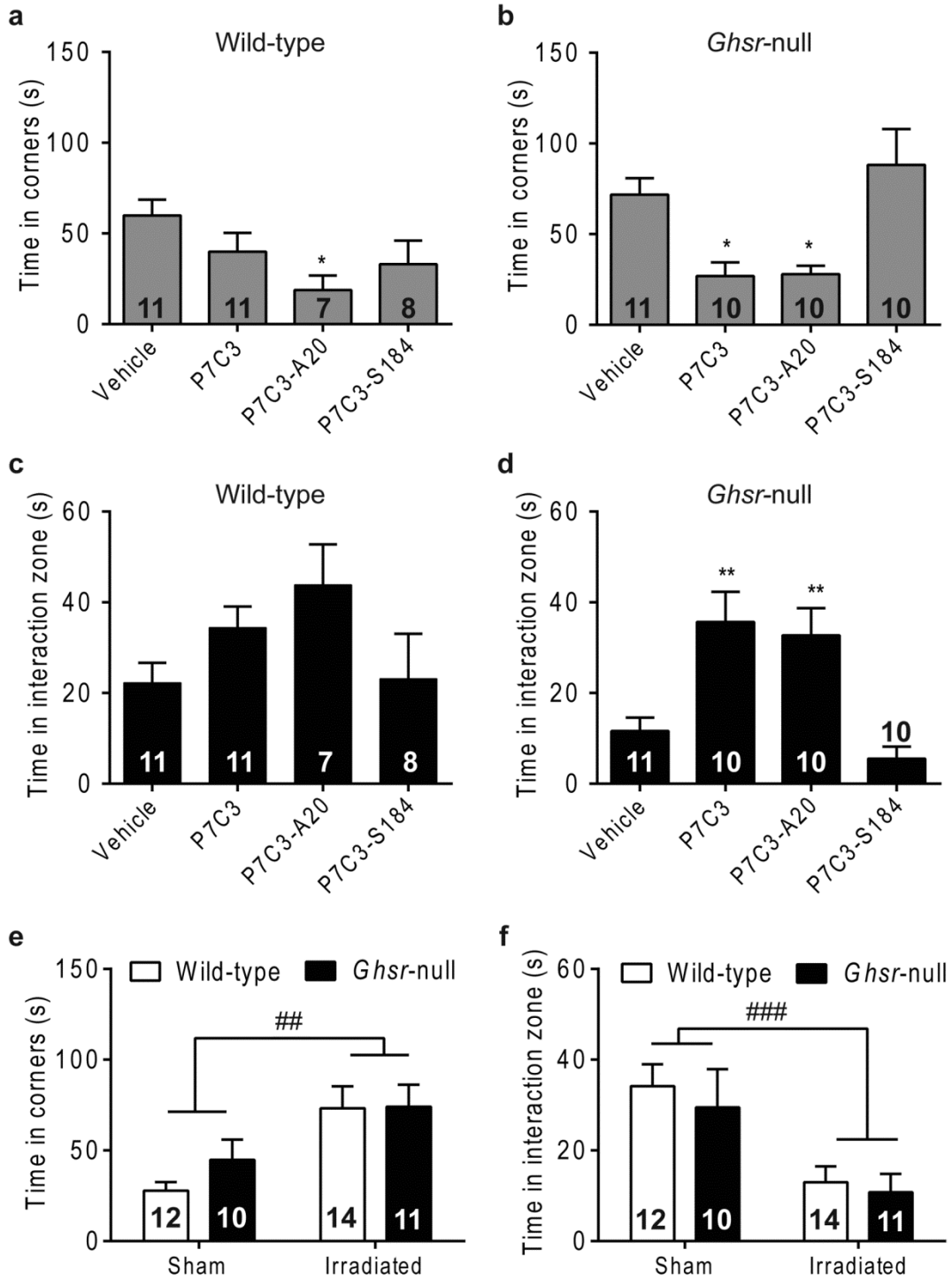

Figure 2.8. Effects of P7C3 on CSDS-induced depressive-like behavior, as measured by the social interaction test. (a,b) Times spent in the corners with target present for wild-type (a) and *Ghsr*-null (b) mice. (c,d) Times spent in the interaction zone by wild-type (c) and *Ghsr*-null (d) mice. Group sizes indicated. (e,f) Times spent in the corners (e) or interaction zone (f) for

sham or irradiated mice. *$P < 0.05$, ** $P < 0.01$, as compared to respective vehicle-treated control group; $^{\#\#}P< 0.01$, $^{\#\#\#}P<0.001$ treatment effect of irradiation.

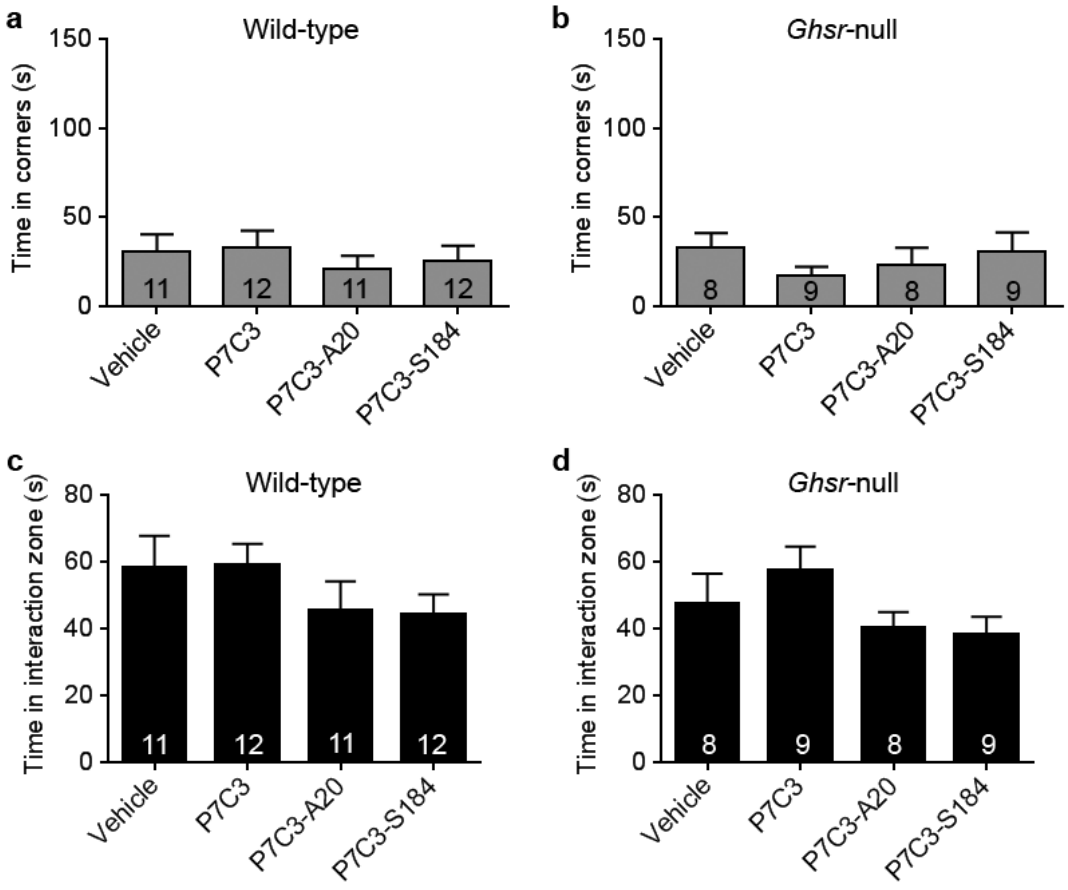

Figure 2.9. Effect of P7C3 compounds on depressive-like behavior in non-CSDS exposed mice. (a-b) Time spent in the corners when the target is present for wild-type (a) and *Ghsr*-null (b) mice. (c-d) Time spent in the social interaction zone when target is present for wild-type (c) and *Ghsr*-null (d) mice. Group sizes as indicated.

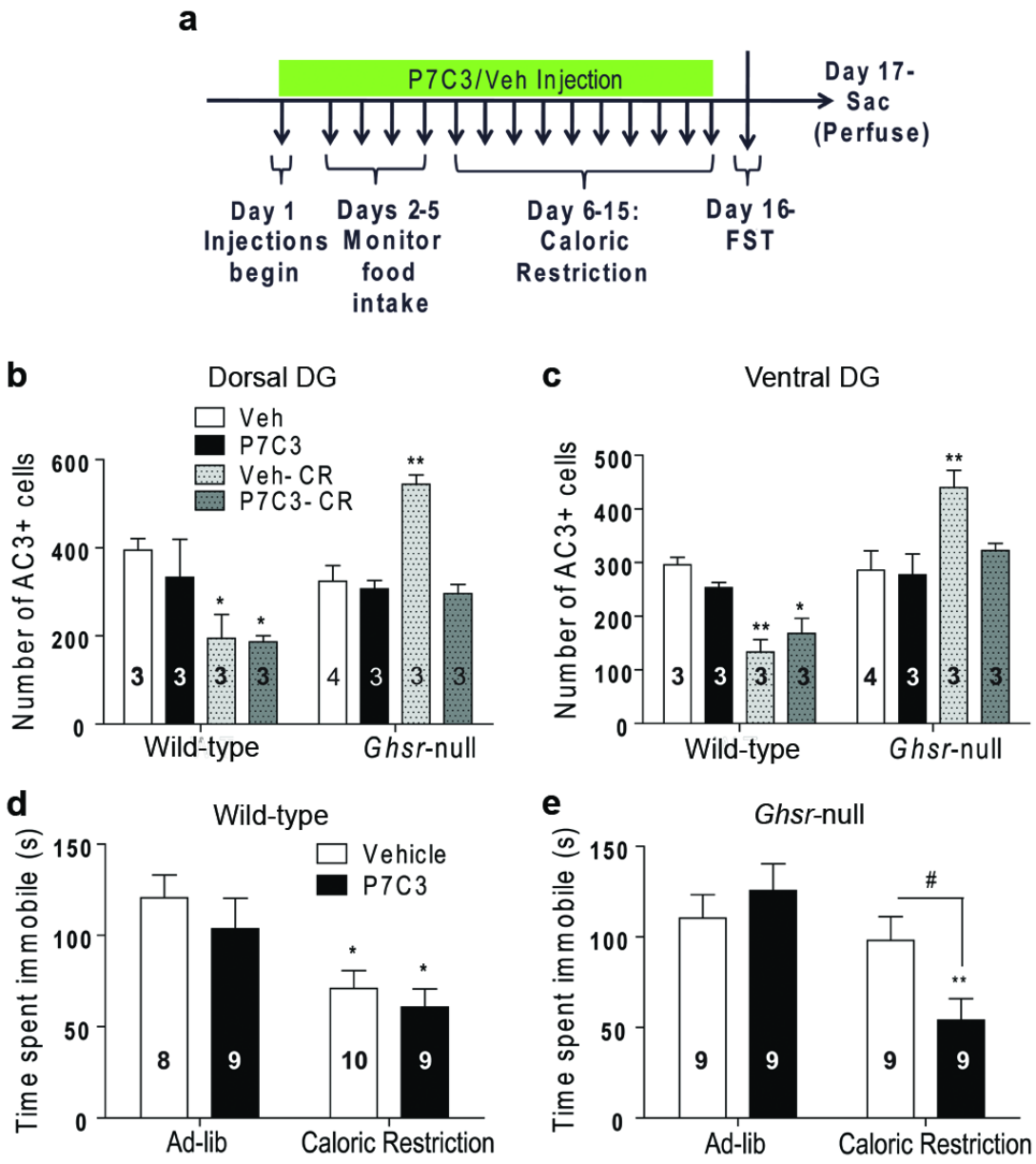

Figure 2.10a-e. Effects of P7C3 on caloric restriction-induced DG cell survival and antidepressant-like behavior, as measured by FST. (a) Protocol schematic. (b-c) AC3-immunoreactive cell counts in the DG of calorie-restricted and *ad libitum*-fed mice treated with P7C3 vs. vehicle. (d,e) FST immobility time. Group sizes indicated. *$P < 0.05$, ** $P < 0.01$, as compared to respective *ad libitum*-fed, vehicle-treated control group. # $P < 0.05$, comparing compound treatment.

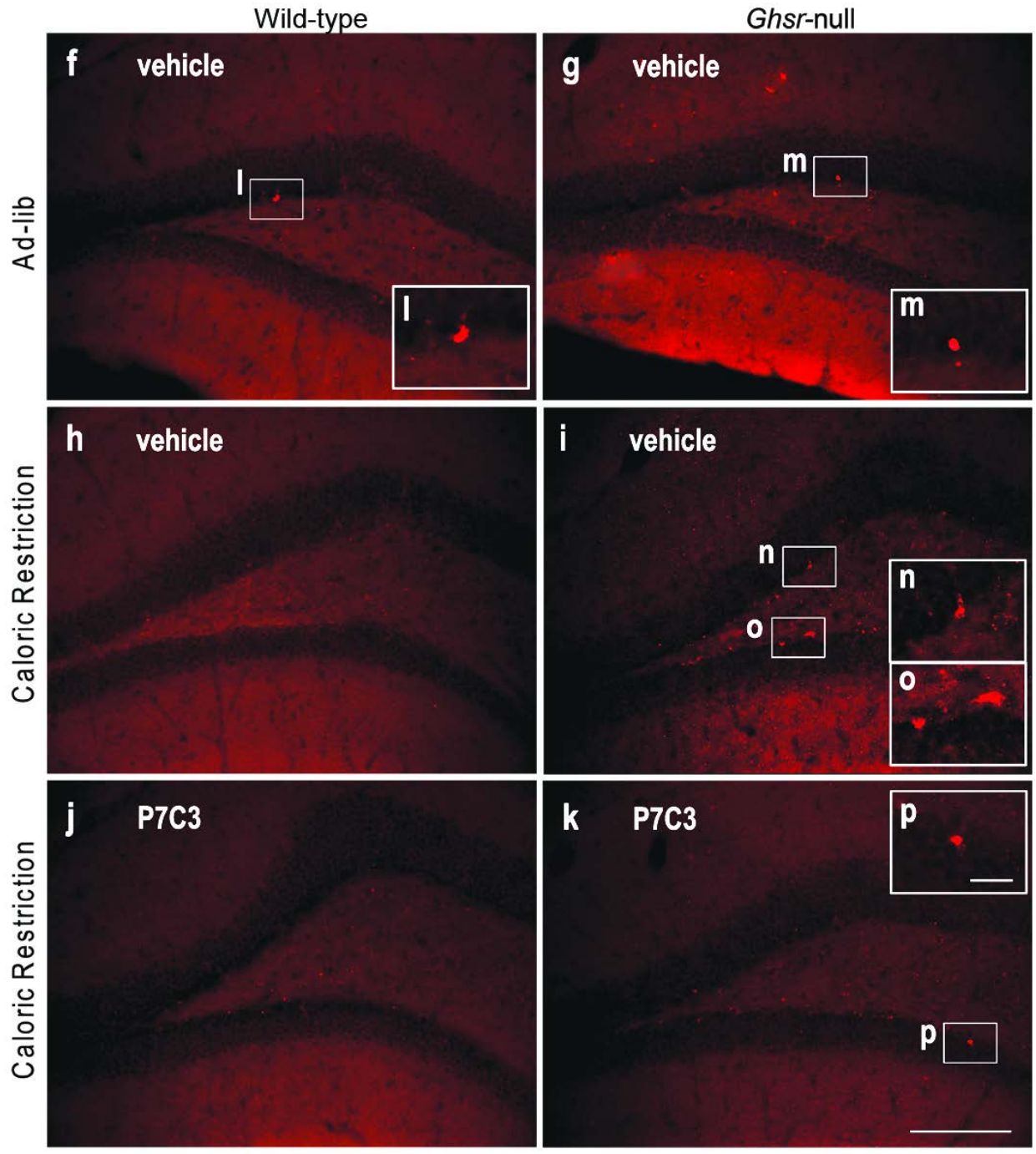

Figure 2.10f-p. Effects of P7C3 on caloric restriction-induced DG cell survival and antidepressant-like behavior, as measured by FST. (f-k) Representative photomicrograph images of AC3-immunolabeled ventral DG sections. (l-p) Magnified images of AC3-immunoreactive cells. Scale bar in (k; 150 µm) pertains to (f-k), in (p; 25 µm) pertains to (l-p).

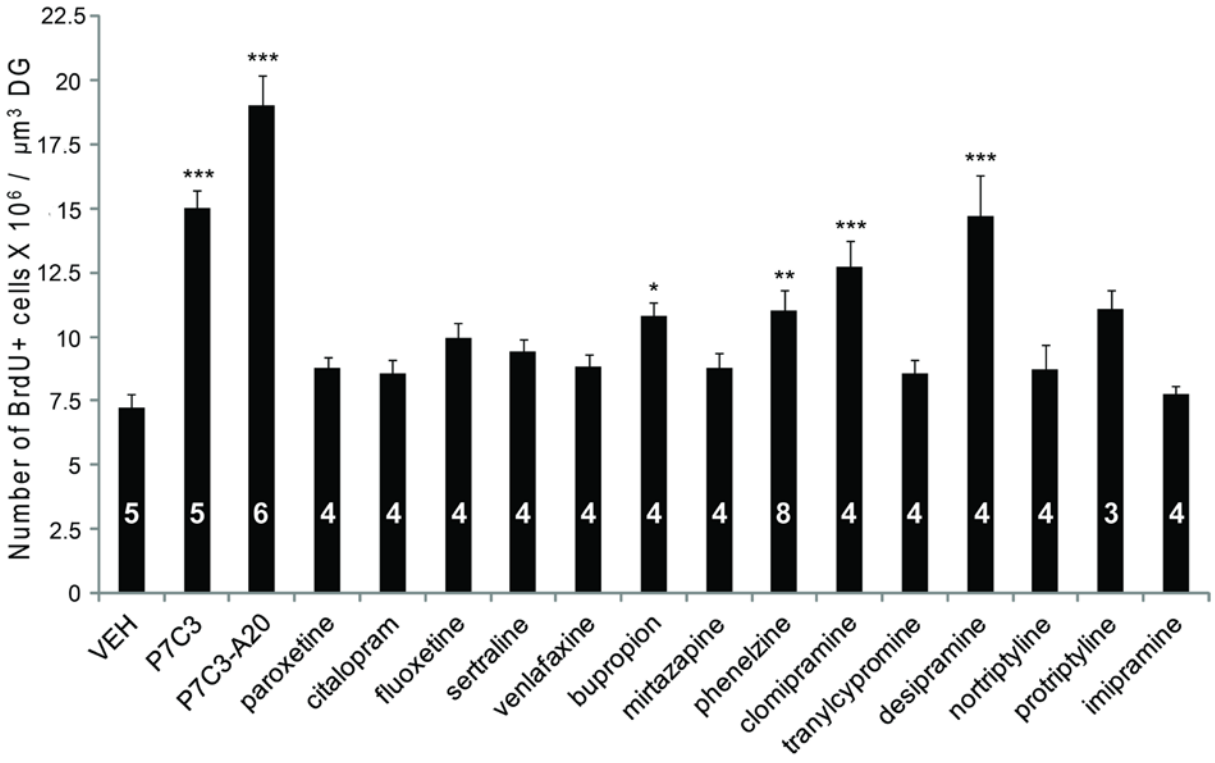

Figure 2.11. Proneurogenic efficacy of P7C3 compounds and antidepressants in DG. BrdU+ DG cell counts in wild-type mice receiving infusions of P7C3 compounds or antidepressants into the left ventricle. Group sizes indicated. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared to vehicle-treated group.

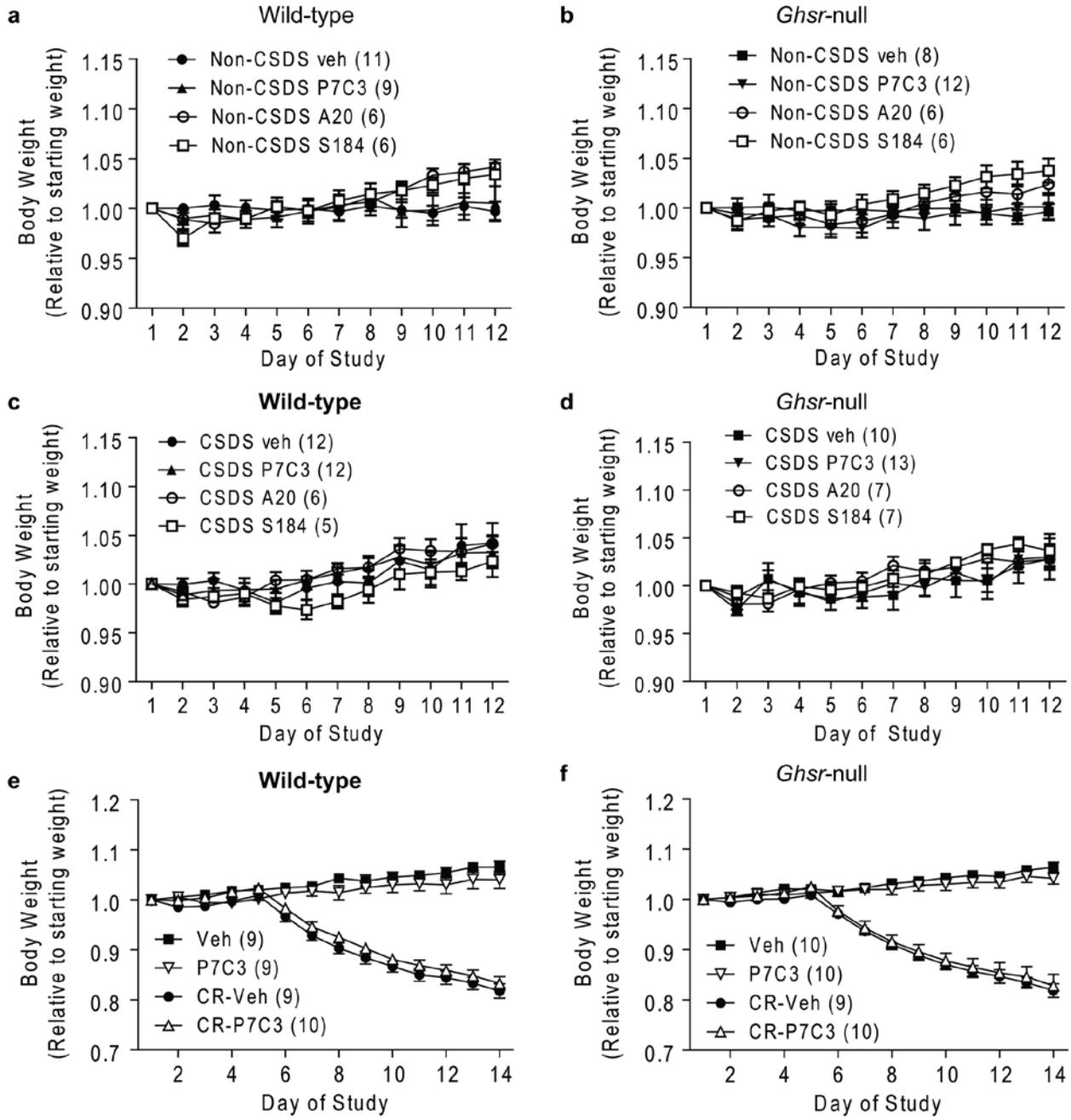

Figure 2.12. Effect of P7C3 compounds on body weight. (a-d) Relative body weights for non-CSDS-exposed wild-type (a) and *Ghsr*-null (b) mice as well as CSDS-exposed wild-type (c) and *Ghsr*-null (d) mice. (e-f) Relative body weights for calorie-restricted (CR) or *ad libitum*-fed wild-type (e) or *Ghsr*-null (f) mice. Group sizes are indicated within parentheses.

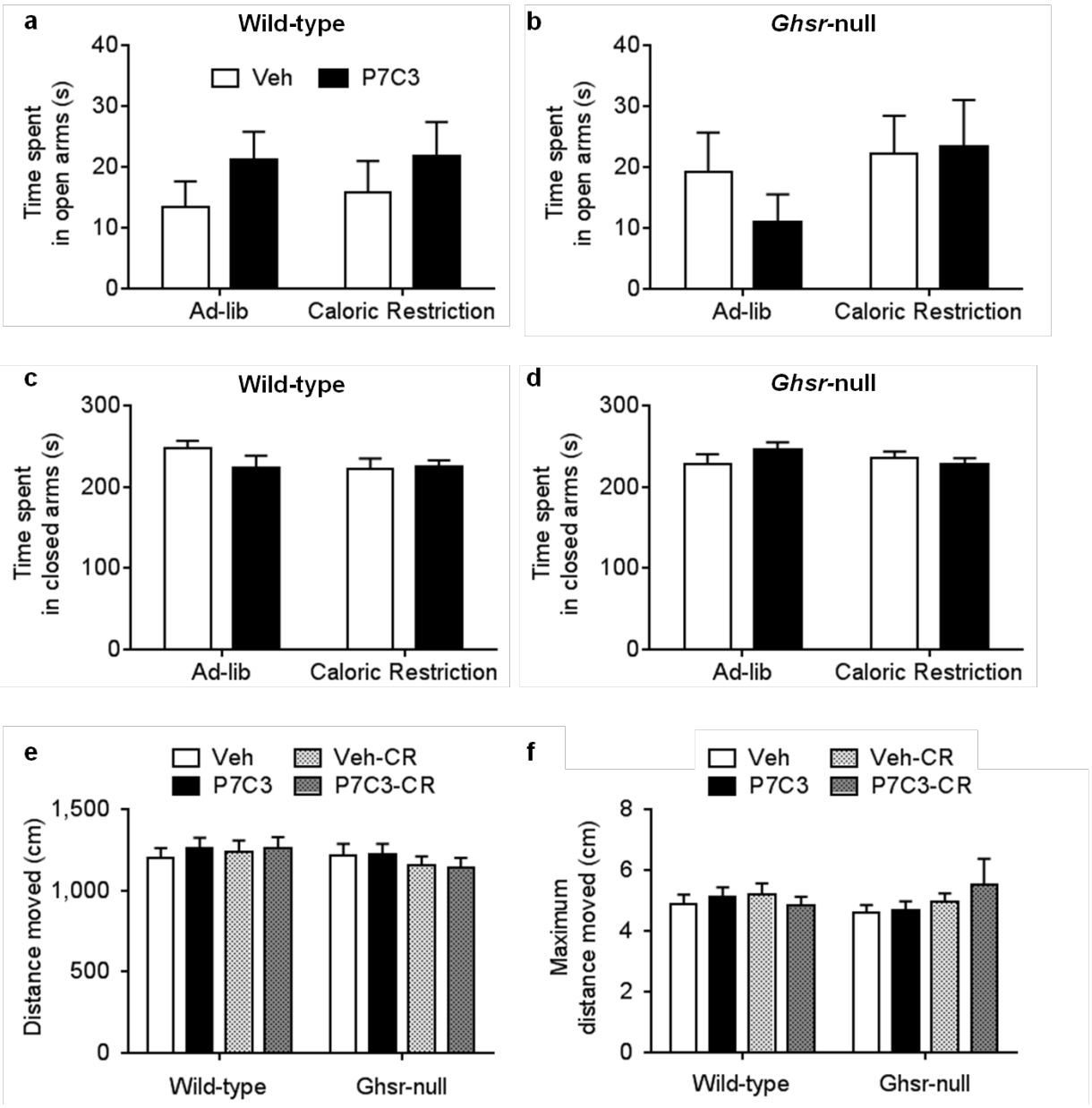

Figure 2.13. Effect of P7C3 or caloric restriction on anxiety related behavior in the elevated plus maze. (a-b) Time spent in the open arms in ad-libitum fed (ad-lib) or calorically restricted (CR) wild-type (a) and *Ghsr*-null (b) mice. (c-d) Time spent in the closed arms in ad-lib fed or CR wild-type (c) and *Ghsr*-null (d) mice. (e-f) Distance moved (e) or maximum distance made in one movement (f) for ad-lib fed or CR mice administered either vehicle or P7C3. N=8-10 mice/group.

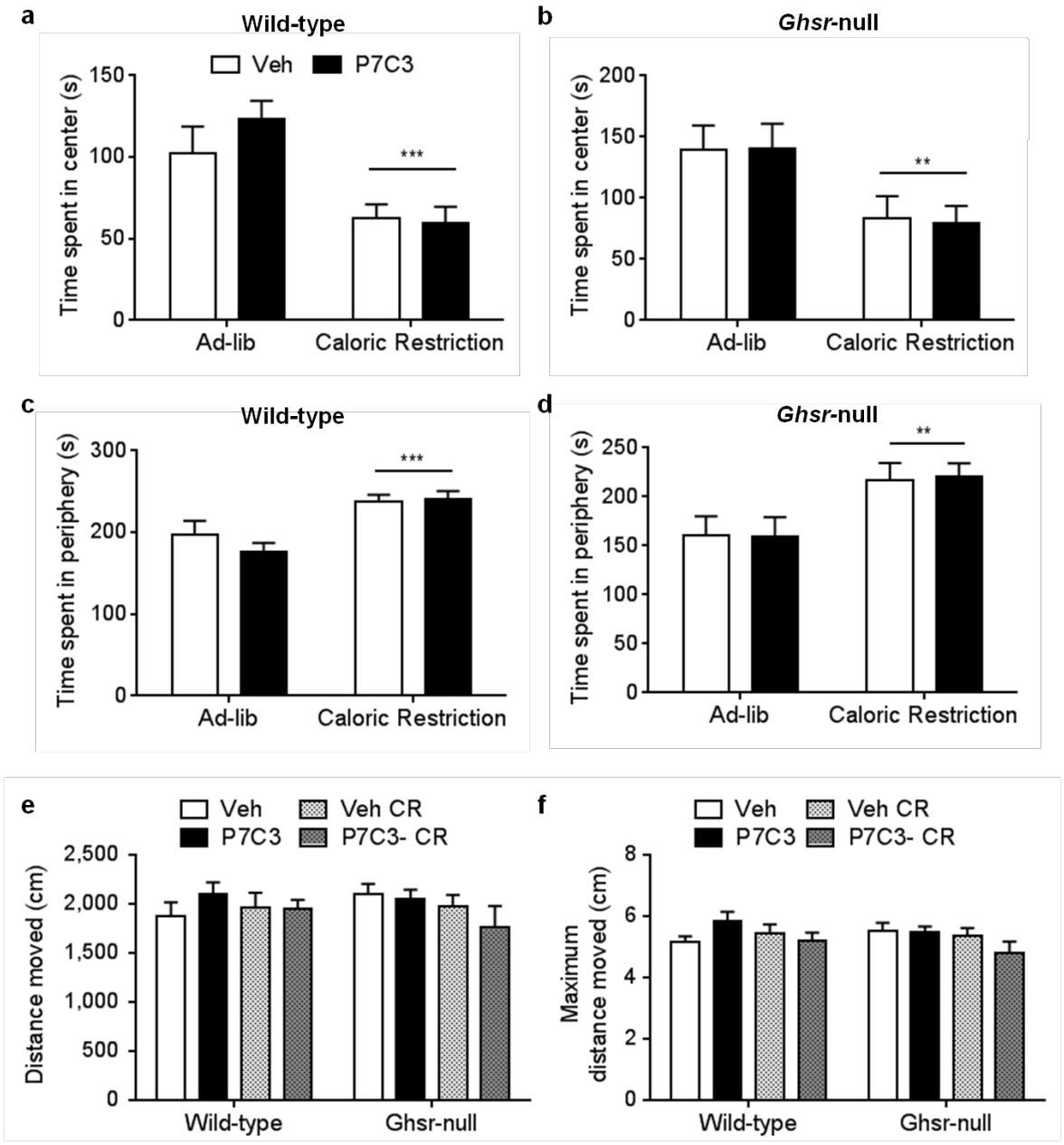

Figure 2.14. Effect of P7C3 and caloric restriction on anxiety related behavior in the open field. (a-b) Time spent in the open arms in ad-libitum fed (ad-lib) or calorically restricted (CR) wild-type (a) and *Ghsr*-null (b) mice. (c-d) Time spent in the closed arms in ad-lib fed or CR wild-type (c) and *Ghsr*-null (d) mice. (e-f) Distance moved (e) or maximum distance made in one movement (f) for ad-lib fed or CR mice administered either vehicle or P7C3. N=8-10 mice/group. $^{**}P < 0.01$, $^{***}P < 0.001$ significant effect of feeding method using a two-way ANOVA.

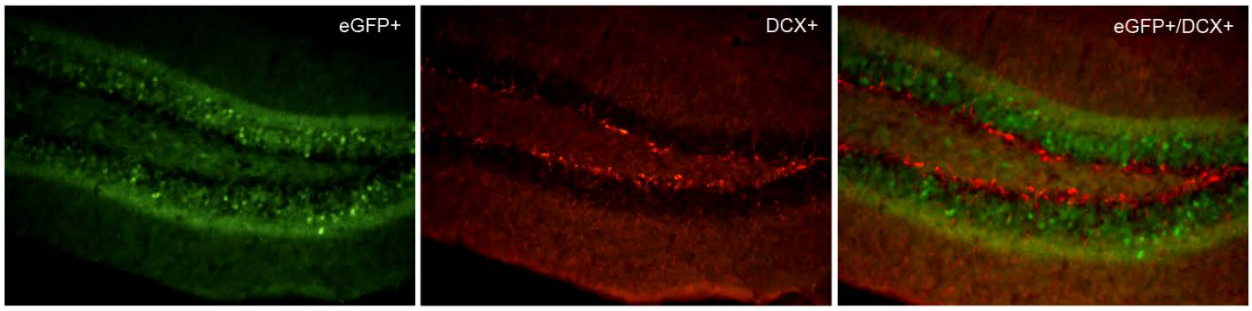

Figure 2.15. Coexpression of eGFP-immunofluorescence (eGFP+) and DCX-immunolabeling (DCX+) in *Ghsr*-eGFP mice.

Photomicrograph images taken of endogenous eGFP, immunolabeling of DCX, and an overlay of the two are represented in the above panels, respectively.

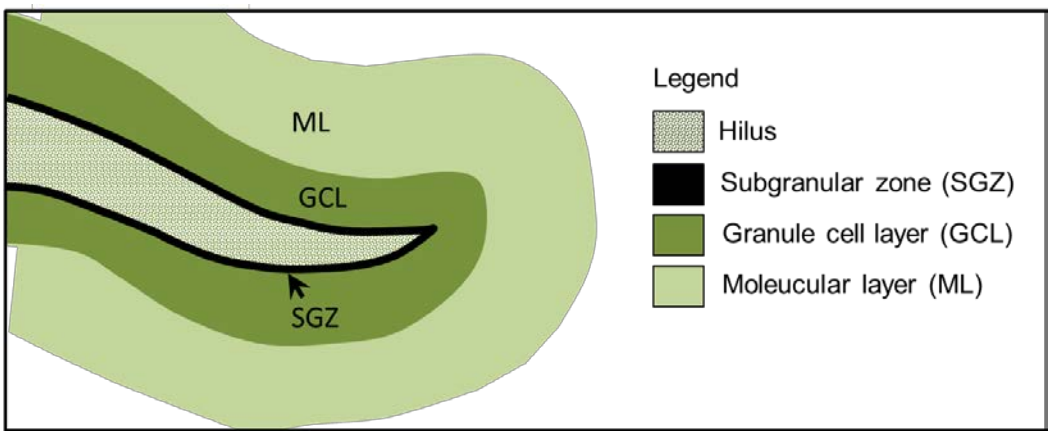

Figure 2.16. Representative diagram of DG subregions.

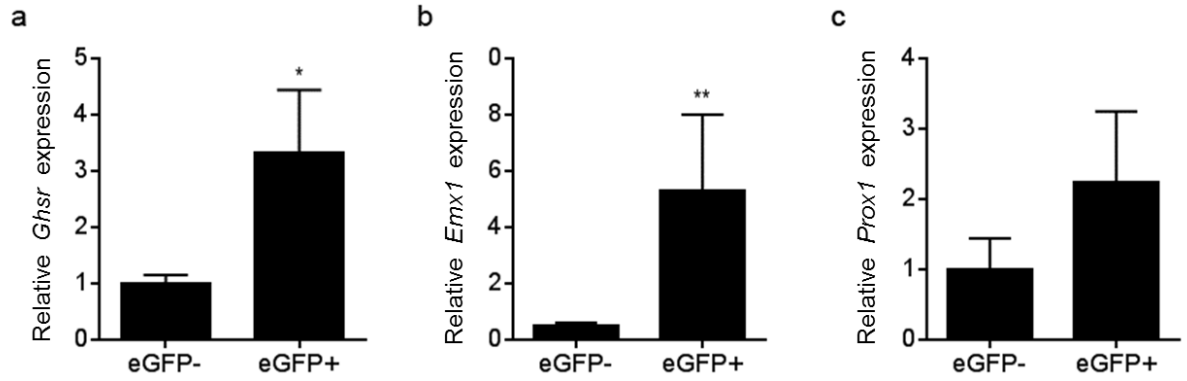

Figure 2.17. Expression of genes associated with DG proliferation in eGFP-enriched (eGFP+) or eGFP-negative (eGFP-) DG cell populations.

(a-c) Relative mRNA expression of Ghsr (a), Emx1 (b), or Prox1 (c) in FAC-sorted eGFP+ and eGFP- DG cell populations. Cyclophilin was used as the internal control. *$P < 0.05$, ** $P < 0.01$, as determined by t-test. (N=5 in a-b, N=3 in c)

CHAPTER THREE

Disruption of cue-potentiated feeding in mice with blocked ghrelin signaling

Adapted from: **Walker AK**, Ibia IE, Zigman JM. Disruption of cue-potentiated feeding in mice with blocked ghrelin signaling. (2012). Physiology & Behavior. 108: 34-43.

Abstract

The peptide hormone ghrelin regulates a variety of eating behaviors. Not only does it potently increase intake of freely-available food, but it also shifts food preference towards diets rich in fat, enhances operant responding for food rewards, and induces conditioned place preference for food rewards. Here, it was postulated that ghrelin also enables cue-potentiated feeding, in which eating is enhanced upon presentation of a food-conditioned stimulus. To test this hypothesis, a novel cue-potentiated feeding protocol adapted for use in mice was designed and validated, and then the effects of pharmacologic ghrelin receptor (GHSR) antagonism and GHSR transcriptional blockade (as occurs in GHSR-null mice) were assessed. Sated C57BL/6J mice indeed demonstrated cue-potentiated intake of grain-based pellets specifically upon presentation of a positive conditioned stimulus (CS+) but not a negative conditioned stimulus (CS-). Treatment with a GHSR antagonist blocked potentiated feeding in sated C57BL/6J mice in response to the CS+. In contrast, while GHSR-null mice also lacked a potentiation of feeding specifically in response to the CS+, they displayed an enhanced intake of pellets in response to both the positive and negative conditioned stimuli. The pattern of immediate early gene expression within the basolateral amygdala -- a brain region previously linked to cue-potentiated feeding -- paralleled the observed behavior of these mice, suggesting uncharacteristic activation of the amygdala in response to negative conditioned stimuli in GHSR-null mice as compared to wild-type littermates. Thus, although the observed disruptions in cue-potentiated feeding are

different depending upon whether GHSR activity or GHSR expression is blocked, a key role for GHSRs in establishing a specific positive cue-food association has now been established.

Introduction

The rates of obesity have been steadily and dramatically increasing (Flegal et al., 2012). Understanding the pathways that regulate complex eating behaviors and ultimately disturb homeostatic control of food intake is crucial for the development of effective obesity treatments. While genetic factors undoubtedly contribute to obesity, an individual's environment and upbringing are also likely involved (Harris et al., 2009; Zimmerman et al., 2010; Saelens et al., 2012). The human environment is replete with visual, auditory, and olfactory cues which, via associative learning and Pavlovian conditioning, can become intimately linked to food, resulting in the induction and maintenance of eating (Holland et al., 2005). Prime examples include logos of commercial enterprises that sell food (Cohen, 2008). With continued exposure, these cues can form such a strong association with eating that they may override satiety signals that otherwise would normally lead to eating cessation (Holland et al., 2005). Recurrent exposure to these cues potentially can lead to an overabundance of food intake resulting in an increased risk for obesity. Of note, the motivational salience of food cues as measured by visual attention is greater in obese individuals than in lean subjects, suggesting that higher sensitivity to cues associated with food may contribute to their lack of control over food intake (Castellanos et al., 2009).

The cue-potentiated feeding paradigm models habitual eating that occurs with strong cue associations linked to food. Several studies have found that food-sated rats increase food consumption after presentation of a conditioned stimulus previously paired with food during a period of caloric restriction (Weingarten, 1983; Holland et al., 2003). These elegant studies were performed with bland pellets similar to regular chow, signifying the strength of a

conditioned cue's ability to enhance feeding behavior even without savory taste as a rewarding component. The amygdala and prefrontal cortex play a major role in this behavior as lesions of the basolateral amygdala (BLA) or medial prefrontal cortex (mPFC) in rats abolish the cue-induced potentiation of eating (Holland et al., 2002; Holland et al., 2003; Petrovich et al., 2007; Petrovich et al., 2007) and as connections from the BLA/basomedial amygdala and mPFC to the lateral hypothalamic area in rats are strongly activated by the positive conditioned stimulus (Petrovich et al., 2005). While these studies using rats have determined some of the neural pathways and regional networks involved in cue-potentiated feeding, to our knowledge, this behavioral model has never been performed in mice using a non-savory food, which is an important distinction since a tasty or rewarding food adds another dimension to the learning aspect of conditioning. The use of mice in place of rats in this paradigm will facilitate studies that aim to identify the molecular mediators involved in shaping and activating these neural networks, as mice can be more easily genetically manipulated.

One potential mediator in the development of cue-potentiated feeding is the gastrointestinally-derived peptide hormone ghrelin (Kojima et al., 1999). Ghrelin potently induces intake of freely-available food upon binding to its receptor, the growth hormone secretagogue receptor (GHSR), in regions including the hypothalamus and brainstem, and it is through these pathways that endogenous ghrelin is thought to affect body weight homeostasis (Wren et al., 2001; Faulconbridge et al., 2003; Perello et al., 2012). GHSR localization to the ventral tegmental area (VTA), hippocampus, and amygdala provides evidence that ghrelin also may mediate more complex eating behaviors that involve different aspects of learning, memory, and reward (Guan et al., 1997; Jiang et al., 2006; Zigman et al., 2006). Indeed, several studies have investigated a role for ghrelin in complex eating behaviors. Ghrelin helps to define food preference -- shifting consumption towards sweet diets and those high in fat, and ghrelin also enhances operant responding for sweet and fatty food rewards (Shimbara et al., 2004; Disse et

al., 2010; Perello et al., 2010; Landgren et al., 2011; Skibicka et al., 2012). Furthermore, ghrelin enables acquisition of conditioned place preference for food rewards upon its pharmacologic administration or upon its natural elevation as induced by caloric restriction or psychosocial stress (Perello et al., 2010; Chuang et al., 2011; Disse et al., 2011). Several studies have indicated that blockade of ghrelin action, by pharmacologic blockade of or genetic deletion of GHSRs, blocks many of these same complex eating behaviors (Abizaid et al., 2006; Egecioglu et al., 2010; Perello et al., 2010; Chuang et al., 2011; Skibicka et al., 2012). To our knowledge, only one study, using a Pavlovian-to-instrumental transfer protocol to study motivational incentive learning, has reported an enhancement in reward behavior upon blockade of ghrelin action (Johnson et al., 2009). The ability of ghrelin to enhance performance in tests of behavioral memory also may be relevant to the pathways required for cue-potentiated feeding (Carlini et al., 2004; Diano et al., 2006). Here, the hypothesis is tested that, in addition to its previously-reported effects on homeostatic eating, food preference, and reward-based eating, ghrelin also participates in the development and expression of cue-potentiated feeding as well as the regulation of BLA activity in response to conditioned cues.

Materials and methods

Animals

C57BL/6J mice (Charles River, Wilmington, MA) were used in Experiments 1 and 2. GHSR-null and wild-type littermates, used in Experiments 3 and 4, were generated by breeding mice heterozygous for the GHSR-null allele, obtained after more than 10 generation backcrossing onto a C57BL/6J genetic background (Zigman et al., 2005). All studies were approved by the UTSW Institutional Animal Care and Use Committee.

Primary cue-potentiated feeding paradigm

Conditioning: This protocol (Figure 3.1A, used for Experiments 1, 3, and 4) was modeled after reported cue-potentiated protocols designed for use in rats with standard chow (Holland et al., 2002; Holland et al., 2003; Petrovich et al., 2005; Petrovich et al., 2007). Two-month-old mice, housed 2-3 per cage, were placed on a restricted feeding schedule which provided access to standard chow (Teklad Global Diet #2016 Madison, WI, which provides 3.0 kcal/g of energy and contains 16.4 g% protein, 4.0 g% fat , and 48.5 g% carbohydrates) for 3 ½ hr per day. Such was maintained during a run-in period (Days 1-5) and throughout a "simple" conditioning phase (Days 6-12) and a subsequent "discrimination" conditioning phase (Days 13-26).

Conditioning sessions were performed by placing individual mice into conditioning chambers (Model ENV307A, Med Associates, Inc., St. Albans, VT) just before the 3 ½–hr period of food availability. During the first, "simple" conditioning phase (Days 6-12), daily conditioning sessions were performed by pairing a light cue, which would become the conditioned positive stimulus (CS+), with delivery of a single 14-mg grain-based Dustless Precision Pellet (BioServ, Frenchtown, NJ, which provides 3.6 kcal/g of energy and contains 18.7% protein, 5.6% fat, and 59.1% carbohydrates). The CS+ was assigned to each mouse in a counterbalanced fashion as either the main "house" light (affixed near the ceiling) of the chamber or its central "nose-poke" light (affixed to the lower wall area of the chamber). Cues lasting 2-sec in duration were given at random intervals every 30 - 90 sec. A single food pellet was dispensed immediately after each CS+ into a food hopper using a programmed automatic pellet dispenser. Thirty cues were delivered per each 30 min-long simple conditioning session. During the second, "discrimination" conditioning phase (Days 13-26), daily conditioning sessions were performed using both positive light cues [conditioned positive stimuli (CS+)] and negative light cues [conditioned negative stimuli (CS-)]. A single food pellet was dispensed upon presentation of the CS+; no

food pellet was dispensed upon presentation of the CS-. The CS- was assigned as whichever light cue was not used as the CS+. Twenty CS+ and 20 CS- cues of 2-sec duration each were delivered in random order and at random intervals every 30 – 90 sec during each 40 min-long discrimination conditioning session. When not in the conditioning chambers, mice were housed in their home cages.

Test sessions: During the first three days following completion of the conditioning (Days 27-29), mice were kept in their home cages with *ad lib*-access to standard chow. For Experiment 1, acquisition of cue-potentiated feeding was assessed on Day 30 by placing mice in the conditioning chambers for three 10-min test sessions: a baseline session where no cue was presented (Session 1), a session where only the CS+ was presented (Session 2 or 3), and a session where only the CS- was presented (Session 2 or 3). Ten cues of 2-sec duration were delivered at random intervals every 30 – 90 sec during Sessions 2 and 3. The orders of the CS+ test session and CS- test session were counterbalanced between animals. During these three test sessions, mice had free access to 30 food pellets within the food hopper. Between sessions, mice were placed into their home cages briefly while the pellets remaining were counted.

Cue-potentiated feeding with ghrelin receptor antagonist

For Experiment 2, the above protocol was modified slightly to allow more time for the mice to adapt to receiving an oral gavage of either a ghrelin receptor antagonist or its vehicle prior to each conditioning session (Figure 3.2). As such, the "simple" conditioning phase was extended to two weeks rather than one (Days 6-19), while the "discrimination" conditioning remained two weeks in length (Days 20-33). Also, for this modified protocol, mice were allowed free access to 20-mg grain-based Dustless Precision Pellets (BioServ, which provides 3.35 kcal/g of energy and contains 21.3 g% protein, 2.8 g% fat, and 54 g% carbohydrates) instead of

standard chow during the 3 ½ hr-long daily feeding periods provided in the home cages after each conditioning session. These grain-based pellets were provided ad lib in home cages in the days following the conditioning phases. Neither antagonist nor vehicle was administered during the 5 day run-in period or the 3 days following conditioning.

Ghrelin receptor antagonism was achieved by administering Compound 26 [also known as LXG-9342, and by its chemical name, (6-(4-Fluorophenoxy)-3-{[(3S)-1-isopropylpiperidin-3-yl]methyl}-2-methylquinazolin-4(3H)-one], which was synthesized by the UTSW Synthetic Chemistry Core, based on published protocols (Esler et al., 2007; Rudolph et al., 2007). One hr prior to each conditioning session (Days 6-33), freshly-prepared Compound 26 suspended in PEG400:water (80:20), at a concentration of 30 µg/10 µL was orally administrated via gavage to provide 30 µg per gram body weight; vehicle-treated control mice were similarly treated with 10 µL PEG400:water (80:20) solution per gram body weight. The initial descriptions of Compound 26 demonstrated a relatively potent affinity to GHSR, high bioavailability, moderate clearance, and a very high volume of distribution including high brain penetrance (Esler et al., 2007; Rudolph et al., 2007). Previously, Compound 26 at a gavage dose of 10 µg/g body weight was shown to reduce glucose excursion in a rat i.p. glucose tolerance test by 20% and to reduce body weights of diet-induced obese C57BL/6J mice by about 3-4% over a 9-day period (Rudolph et al., 2007). Compound 26 at a gavage dose of 30 µg/g body weight previously was shown to significantly reduce food intake in C57BL/6J mice subjected to a fasting-refeed protocol, but not in similarly-treated GHSR-null mice, suggesting its specificity for GHSR (Esler et al., 2007; Rudolph et al., 2007; Perello et al., 2010). This same 30 µg/g body weight gavage dose of Compound 26 also previously was shown to block the acquisition of conditioned place preference for high-fat diet in chronically calorie-restricted C57BL/6J mice, but not the compensatory hyperphagia associated with chronic calorie restriction (greater than 1 wk restricted, 4-hr-long daily access to standard chow) (Perello et al., 2010).

The first test day (Day 37) was performed in the same manner as in Experiment 1, without Compound 26 or vehicle. A second test day (Day 38) involved administration of Compound 26 (30 µg/g body weight) or vehicle 1 hr prior to the baseline session. The length and order of test sessions were the same as described for Experiment 1.

Cue-potentiated feeding in GHSR-null mice

Experiment 3 involved the use of GHSR-null mice and wild-type littermates. The protocol was identical to that used in Experiment 1 (Figure 3.1A), except two additional test days were performed on Days 31 and 32 (Figure 3.1B). On Day 31, the same three 10-min sessions from Day 30 were performed on the mice following a 16-hr overnight fast imposed in their home cages. On Day 32, after the mice had again been provided ad lib access to standard chow, cue-potentiated feeding was again assessed, only this time, Sessions 2 and 3 lasted 20 min each.

Cue-potentiated feeding paradigm for gene expression study

For Experiment 4, the above protocol was adjusted for a separate cohort of GHSR-null and wild-type littermates to determine whether the conditioned stimuli acutely activate BLA neurons (Figure 3.1B). A single, alternate test day was performed on Day 30 consisting of three 5-min sessions. In between Sessions 2 and 3, the mice were placed back in their home cages for 25 min in order to match the timing of specific immediate early gene peak expression levels to neuronal activation. Tissue collections occurred directly after Session 3. Control mice consisting of both GHSR-null and wild-type littermates ("no cue" group) underwent Days 1-29 of the protocol, as usual, but did not undergo Day 30 testing; tissue collections were done on Day 30.

Sample collection and quantitative real-time PCR (qPCR)

Mice were euthanized by live decapitation. Brains were extracted, placed in cold diethylpyrocarbonate-PBS, and then sectioned into 1 mm-thick coronal slices by use of a stainless steel mouse brain matrix and standard razor blades. Bilateral tissue punches corresponding to the locations of the BLA, mPFC, and VTA were frozen with liquid nitrogen after being excised using a 15-g needle. Quantitative PCR was performed on total RNA that was extracted and reverse transcribed, as described previously (Kurrasch et al., 2004; Chuang et al., 2011). Arc, Homer1a, c-Fos, GHSR, and cyclophilin primers (Table 1) were previously used and/or validated for the appropriate specificity and efficiency using template titration and dissociation curves (Roloff et al., 2010; Besnard et al., 2011; Chuang et al., 2011) and were designed to span exon-exon junctions. Cyclophilin expression levels were used for normalization, and relative levels were calculated by the comparative threshold cycle (ΔΔCt) method, with comparison of the wild-type control (no-cue) group as the baseline.

Statistical Analysis

A one way ANOVA was performed when analyzing the effect of cue on pellet consumption (Experiment 1). Repeated measures two way ANOVA was performed when analyzing the effects of administered compound (Experiment 2) or genotype (Experiment 3) and cue on pellet consumption. Two way ANOVAs were performed when analyzing the effects of genotype and cue presentation on relative immediate early gene mRNA expression (Experiment 4). Bonferroni post-hoc analysis was used for all comparisons with significant *P* values. Linear regression analysis was performed on correlation plots for gene expression and pellet consumption, allowing the determination of coefficients of determination (R^2) for the goodness of fit; slopes that were statistically significantly different from a slope of zero were considered as

indicating significant correlations (Experiment 4). Data are expressed as mean ± SEM, with $P < 0.05$ considered statistically significant. Analyses were performed using GraphPad Prism 5.0.

Results

Experiment 1: Cue-potentiated feeding of grain-based pellets in mice

A cue-potentiated feeding protocol (Figure 3.1) was adapted for use in mice in order to recapitulate the behavior observed by others in rats. These adaptations mostly reflect the slower learning curve of mice and were done to ensure adequate conditioning. Male C57BL/6J mice were first subjected to a 5-day run-in period during which home cage access to standard chow was made available for 3 ½ hrs per day. Next, while still on this caloric restriction regimen, mice were subjected to a "simple" conditioning session on 7 successive days followed by a "discrimination" conditioning session on 14 successive days. Conditioning sessions were performed in special chambers just prior to the 3 ½ hrs of daily food availability in the home cages. During these conditioning sessions, a single14-g grain-based pellet was dispensed into a food hopper upon the randomly-timed presentation of each positive conditioned stimulus (CS+), whereas no pellet was dispensed upon the randomly-timed presentation of negative conditioned stimuli (CS-). The stimuli consisted of either the chamber's ceilinged house light or its mural central nose-poke light. As described more fully in Materials and Methods, the "simple" conditioning sessions included CS+ cues whereas the "discrimination" conditioning sessions included a mixture of CS+ and CS- cues. Following the conditioning period, mice were given ad lib access to standard chow in their home cages and subsequently were tested for the acquisition of cue-potentiated feeding.

Caloric restriction reduced body weights by 18.8 ± 0.5% by Day 6, which persisted throughout the conditioning period (Figure 3.3A). A similar, 4-hr per day restricted food availability protocol previously was shown to stimulate a two-fold elevation in circulating acyl-ghrelin (Perello et al., 2010). By Day 3, the mice had learned to eat all of the pellets dispensed into the food hopper (Figure 3.3B). After completing both conditioning phases, the mice regained their original weights after only two days of ad lib feeding, signifying a presumed sated state (Figure 3.3A).

The primary outcome of the study was assessing the acquisition of cue-potentiated feeding. Indeed, on Day 30, the CS+ induced a specific potentiation of feeding as compared to baseline and the CS-, as indicated by the 2-fold increase in number of pellets consumed (Figure 3.3C). Thus, even without a savory component to the food, mice are capable of forming strong specific cue-food associations that manifest in the sated state.

Experiment 2: Effects of ghrelin receptor antagonism on cue-potentiated feeding

To assess the role of ghrelin in mediating the development of the cue-food associations, the cue-potentiated feeding experiment was performed using a GHSR antagonist, Compound 26 (Esler et al., 2007; Rudolph et al., 2007; Perello et al., 2010). Caloric restriction resulted in reductions of 16.6 ± 0.5% (vehicle-treated group) and 17.2 ± 0.4% (Compound 26-treated group), with no statistically-significant differences between the groups (Figure 3.4A). Although Compound 26 initially was associated with an increased number of pellets leftover after conditioning sessions as compared to vehicle during the majority of the “simple” conditioning phase, throughout the “discrimination” conditioning phase, both groups were eating all of the pellets presented during the conditioning sessions (Figure 3.4B).

As expected based on the Experiment 1 results, mice receiving vehicle displayed a significant enhancement of food intake in response to the CS+ as compared to baseline and the CS-. In contrast, Compound 26 given during the conditioning period only but not on the test day completely blocked the acquisition of cue-potentiated feeding, as the number of pellets eaten was not enhanced by the positive cue (Figure 3.4C). The same was true when Compound 26 was also given on the test day (Figure 3.4D).

Experiment 3: Effects of deleted GHSR expression on cue-potentiated feeding

In an attempt to corroborate the findings with Compound 26, GHSR-null mice were utilized, which lack ghrelin receptor expression, in the cue-potentiated feeding paradigm (Zigman et al., 2005). GHSR-null and wild-type littermates were calorically restricted and conditioned for cue-potentiated feeding. Mice of both genotypes lost 16.4 ± 0.2% of their original body weights upon caloric restriction (Figure 3.5A). In contrast to Experiment 2, mice of both genotypes quickly learned to eat all the pellets dispensed during the conditioning sessions, with no difference between genotypes (data not shown).

Similar to previous experiments, wild-type mice ate significantly more during the CS+ session than during the CS- or baseline sessions (Figure 3.5B). GHSR-null mice instead increased their intake of pellets with both the CS+ and the CS- cues (Figure 3.5B).

Slightly modified tests of cue-potentiated feeding were performed over the next two days. On Day 31, the mice were assessed after first having been fasted in their home cages overnight. Among many other changes, such overnight fasting is known to raise plasma acyl-ghrelin levels (Perello et al., 2010) although it is presumed that only wild-type mice with intact GHSR expression, and not GHSR-null mice, can respond to this elevated acyl-ghrelin. Both wild-type and GHSR-null littermates ate more pellets during the baseline session (in the

absence of either the CS+ or CS-), as compared to during the previous Day 30 baseline session, as expected since this was their first exposure to food since the overnight fast began 16 h earlier (Figure 3.5C). Furthermore, wild-type mice maintained an increase of food intake with presentation of the CS+ as compared to the CS-, although not compared to baseline (Figure 3.5C). GHSR-null mice again displayed no significant difference in the amount eaten between the CS+ and CS- test sessions. Upon prolongation of the test sessions to 20 min each (Day 32) using mice under ad lib-fed conditions, wild-type mice again ate significantly more pellets during the CS+ session than the CS- session. GHSR-null mice showed an elevated intake of pellets during both cue sessions as compared to baseline, with no significant difference in pellets eaten between the two cue sessions (Figure 3.5D).

Experiment 4: Effect of deleted GHSR expression on BLA activity in response to cues

A directed examination of GHSR gene expression within the mPFC and BLA was performed given their known roles in cue-potentiated feeding (Holland et al., 2002; Holland et al., 2003; Petrovich et al., 2005). GHSR levels in BLA brain punches were on par with those in the VTA, which is a region well-characterized for GHSR expression, while the mPFC displayed a lack of significant GHSR expression, confirming previous reports (Abizaid et al., 2006; Zigman et al., 2006; Chuang et al., 2011) (Figure 3.6A). Corroborating evidence also revealed eGFP expression in the BLA of mice in which eGFP expression is under the control of the GHSR promotor (Figure 3.7).

Given the observations that GHSR-null mice responded with a non-specific increase of feeding in response to both positive and negative cues, the activity of the BLA in response to the cues was next examined. In order determine whether BLA neuronal activation was specific to the CS+, expression of a set of immediate early genes was examined using a protocol previously used in rats (Petrovich et al., 2005). An alternate version of the Day 30 Test

Sessions was employed, with the timing of the sessions designed such that the immediate early gene qPCR data would inform the timing of neuronal activation. In particular, this method relies on differential, unique peak mRNA expression signatures for the tested immediate early genes in relation to neuronal activation: c-Fos and Arc are not expressed at high levels until about 30 min after neuronal activation, while Homer1a is expressed highly at about 5 min following neuronal activation but is degraded by 30 min (Vazdarjanova et al., 2002; Kelly et al., 2003; Knapska et al., 2007; Day et al., 2008). By spacing the CS+ test and CS- test 25 min apart from each other (with the order still counterbalanced) and obtaining BLA brain punches from mice directly following Test Session 3, it is possible to determine whether neurons are activated by the Session 2 cue or by the Session 3 cue by assessing mRNA levels for these immediate early genes (Figure 3.6B). As such, elevated Homer1a levels (as compared to control mice given no cues) indicate neuronal activation by the Session 3 cue (second cue); elevated Arc and c-Fos levels indicate neuronal activation by the Session 2 cue (first cue).

In wild-type mice, elevated BLA Homer 1a expression did not occur when the CS+ was exhibited during Session 2 (CS+ first) but was observed when the CS+ was exhibited during Session 3 (CS+ second), suggesting specific BLA neuronal activation in response to the CS+ but not the CS- (Figure 3.6C). Supporting these data, in wild-type mice, c-Fos and Arc levels were both increased when the CS+ was tested first but not when the CS- was tested first (Figures 3.6E,G). In contrast, GHSR-null mice demonstrated elevated Homer1a regardless of which cue was first. Similarly, in GHSR-null mice, both c-Fos and Arc levels were elevated when either the CS+ or CS- was tested first. These data suggest BLA neuronal activation by both cues in GHSR-null mice.

Immediate early gene expression within the BLA of GHSR-null and wild-type littermates was also compared to the number of pellets eaten by these animals during Test Sessions 2 and

3. Relative expression of Homer1a positively correlated with the number of pellets eaten by the mice during Session 3, while the relative expression of c-Fos positively correlated with the number of pellets eaten during Session 2 (Figures 3.6D, F). No statistically significant correlation between the relative expression of Arc and the number of pellets eaten during Session 2 was observed.

Discussion

In the present study, the design and validation of a cue-potentiated feeding protocol for enhanced consumption of a non-savory food in mice is described. Previous studies using the cue-potentiated feeding paradigm in rats, on which the current mouse protocol was modeled, have already provided some key insights into the neural pathways regulating this habitual eating behavior. A related behavioral model, Pavlovian-Instrumental Transfer, which assesses the willingness to lever press in response to a cue previously paired with a rewarding food substance, has been reported for mice in addition to rats (Johnson et al., 2009; O'Connor et al., 2010; Lederle et al., 2011). The new mouse protocol described here reduces the complexity of these previous Pavlovian Instrumental Transfer studies to focus primarily on the importance of developing a cue-food association rather than motivation. This protocol also successfully employs the use of non-savory, bland food pellets for the cue-potentiated feeding design, as in the classic studies with rats, so as to avoid adding the additional component of a food reward that has a naturally high hedonic value. Thus, the success of this design permits future opportunities to tease apart any distinctions between molecular mediators involved in the simple cue-induced potentiation of food intake as opposed to potentiated intake of food rewards with a high hedonic value.

After validating the appropriate cue-potentiated feeding response for grain-based pellets in C57BL/6J mice, the involvement of ghrelin signaling in this behavior was assessed due to ghrelin's numerous roles in other feeding behaviors. Given ghrelin's previously described roles in stimulating intake of freely-available food and multiple reward-based eating behaviors – it was predicted that neither the CS+ nor the CS- would potentiate feeding. As predicted, upon administration of a GHSR antagonist before each conditioning session, mice no longer displayed a potentiation of food intake in response to cues, regardless of whether the antagonist was additionally given prior to the test sessions or not. Such suggests the requirement for intact ghrelin signaling in the acquisition of the cue potentiated feeding behavior.

A potential caveat in the interpretation of the GHSR antagonist studies is that during the initial "simple" conditioning phase, mice receiving Compound 26 seemed slower to adapt to the conditioning and failed to consume all of the pellets provided with the CS+. That said, by the "discrimination" phase of training, mice in both the vehicle-treated and Compound 26-treated groups were eating all or nearly all of the pellets provided during each conditioning session. Therefore, it is probable that both groups consumed enough pellets during the "discrimination" sessions to have received appropriate conditioning to the cues.

As a means of corroborating the effects of pharmacologic blockade of ghrelin action on cue-potentiated feeding, the performance of mice lacking GHSRs was next compared with that of wild-type littermates in the cue-potentiated feeding protocol. Utilizing GHSR-null mice and wild-type littermates, it was determined that lack of GHSRs disrupts the specific potentiation of eating in response to a CS+ that is otherwise exhibited by wild-type mice. However, while a similar response was expected in mice lacking GHSRs and mice receiving Compound 26 (no potentiation of feeding with either the CS+ or the CS- cues), instead both the CS+ and the CS- potentiated feeding. While the reasons for this observed behavior are not yet clear, it does

seem that GHSR-null mice form an abnormal association with the negative cue, resulting in enhanced food intake. It could be that GHSR-null mice have a harder time learning the specific association of a single cue with receiving food and automatically associate any cue in that particular environmental context with the receipt of food. Therefore, the GHSR-null mice may simply respond to any change in the environment, such as the short presentations of light, with an increase in food intake. Otherwise stated, the GHSR-null mice may have difficulties discriminating the two discrete cues, and therefore, cannot distinguish between the two as separate prompts.

Failure of GHSR-null mice to discriminate the CS+ and CS- cues makes sense from the standpoint of most of the reported effects of ghrelin on learning and memory. Learning deficits might also have contributed to the prolonged number of conditioning sessions taken by Compound 26-treated C57BL/6J mice to finish all of the pellets provided upon presentation of the CS+ during conditioning. .Previously, ghrelin-KO mice were shown to perform poorly in tests of behavioral memory such as novel object recognition, while ghrelin administration reverses these deficits (Diano et al., 2006). Upon chronic caloric restriction, administration of ghrelin to supraphysiological levels improves novel object recognition in wild-type mice (Carlini et al., 2008). Direct microinjection of ghrelin into the hippocampus, which is a well-known learning and memory regulatory region, as well as into the amygdala, dose-dependently increase memory retention in wild-type mice (Carlini et al., 2004). Also, GHSR-KO mice have deficits in contextual fear conditioning, which is mediated by the amygdala (Goosens et al., 2001; Albarran-Zeckler et al., 2012). Performance of GHSR-KO mice in the Morris water maze has been mixed, with one study showing reduced and another showing improved spatial learning (Davis et al., 2011; Albarran-Zeckler et al., 2012). Regarding the hippocampus, GHSRs are found throughout all its regions, peripherally-administered radiolabeled ghrelin is taken up by the hippocampus, and ghrelin can increase hippocampal spine synapse density (Diano et al.,

2006). These findings are thought may be relevant to cue-potentiated feeding since a strong learning component is involved in the development of specific cue-food associations and reduced learning capacity may cause problems with cue discrimination.

Notwithstanding the above-described GHSR expression in and ghrelin action at the hippocampus, previous lesioning studies in rats have demonstrated that the BLA and mPFC are required for the cue-potentiated feeding response (Holland et al., 2002; Petrovich et al., 2007). Thus the effects of GHSR deletion on cue-potentiated feeding likely also reflect direct and/or indirect effects of ghrelin at the BLA and/or mPFC. Any direct effects of ghrelin would necessitate GHSR expression at those sites. Here, GHSR expression was indeed localized to the BLA but not to the mPFC, using qPCR methodology. Of note, the lab's previous in situ hybridization histochemistry characterization study of GHSR expression in the rat and mouse brains did not reveal amygdala expression (Zigman et al., 2006), although this likely underrepresents the actual amygdala GHSR expression and is in contrast to several subsequent studies: GHSR expression has been localized to the rat amygdala by qPCR (Landgren et al., 2011); GHSR-IRES-tauGFP and GHSR-eGFP reporter mice display fluorescent signal in the amygdala (Jiang et al., 2006; Spencer et al., 2012); β-galactosidase expression occurs in the anterior cortical amygdala in GHSR-KO mice in which LacZ-reporter gene expression marks the sites of usual GHSR expression (Tong et al., 2011). Importantly, ghrelin injection into the BLA increases memory retention in rats, and in humans, ghrelin modulates the amygdala in response to food cues (Malik et al., 2008; Goshadrou et al., 2012).

While GHSR localization to the BLA does not prove that direct ghrelin action at the amygdala mediates ghrelin's effects on cue-potentiated feeding, it was hypothesized that the abnormal behavioral responses of GHSR-null mice upon testing in the cue-potentiated feeding paradigm would manifest in the BLA. More specifically, it was questioned whether BLA

neuronal activation in response to the cues would parallel the cue-potentiated feeding behavior observed in wild-type and GHSR-null mice. Similar to previous studies with rats which demonstrated specific activation of BLA/basomedial amygdala neurons in response to a CS+ (Petrovich et al., 2005), here, elevations in Homer1a, Arc, and c-Fos mRNA levels in wild-type mice correlated with the timing of presumed neuronal activation during the CS+ test session and not the CS- session. In GHSR-null mice, the pattern of immediate early gene mRNA elevations correlated with BLA neuronal activation following presentation of both the CS+ and CS- cues. This suggests a non-discriminatory activation of the BLA in GHSR-null mice in response to cues, supporting the notion that GHSR deletion prevents formation of a specific association with the CS+, as otherwise occurs in wild-type mice. Importantly, the relative levels of Homer1a and c-Fos expression correlated with the number of pellets eaten in GHSR-null and wild-type mice, verifying the importance of the BLA in the expression of cue-potentiated feeding. Future studies should include those that can definitively confirm the requirement and sufficiency for GHSR expression in the BLA and/or other sites, such as the hippocampus or VTA, for ghrelin's effects on cue-potentiated feeding. .

While it is surprising that genetic GHSR deletion resulted in a potentiation of feeding with both cues rather than the complete absence of potentiated feeding as in mice receiving Compound 26, such may provide insight as to differences between life-long absence of GHSR expression and pharmacologic competitive GHSR antagonism in adults. A caveat of the GHSR-null mouse model is that these mice have developed without the presence of GHSR expression from inception. Such may impact neurodevelopment including that of compensatory pathways, which in turn could influence the cue-potentiated feeding behavior investigated here (Steculorum et al., 2011). Fundamental differences between the two methods employed here to alter ghrelin/GHSR signaling may also be a result of differential effects of these methods on GHSR interactions with other cell surface receptors. As a prime example, recent developments

into GHSR function have revealed the formation of heteromers between GHSR and subtype-2 dopamine receptors, and it was found that the presence of GHSRs influence D2 receptors independently of actual GHSR activity (Kern et al., 2012).

Conclusions

The multitude of food-related cues to which humans are exposed may very well contribute to the obesity epidemic. The cue-potentiated feeding model is a useful tool for studying the neuroanatomical circuitry and molecular mechanisms that contribute to habitual eating behaviors. Blockade of ghrelin action by either GHSR antagonist administration or genetic GHSR deletion both disrupt the development of the usual cue-potentiated feeding response. However, while GHSR antagonist blocks potentiated feeding specifically in response to a positive conditioned stimulus, life-long GHSR deletion results in non-specific cue-food associations, as evidenced by potentiated feeding in response to both positive and negative conditioned stimuli. Although the sources of the observed discrepancies are unclear, these studies nonetheless demonstrate the importance of intact ghrelin signaling in the development of cue-potentiated feeding. Further studies are needed to better understand the degree to which and the mechanisms by which ghrelin influences this important feeding behavioral response.

Conflict of Interest: The authors declare no conflict of interest.

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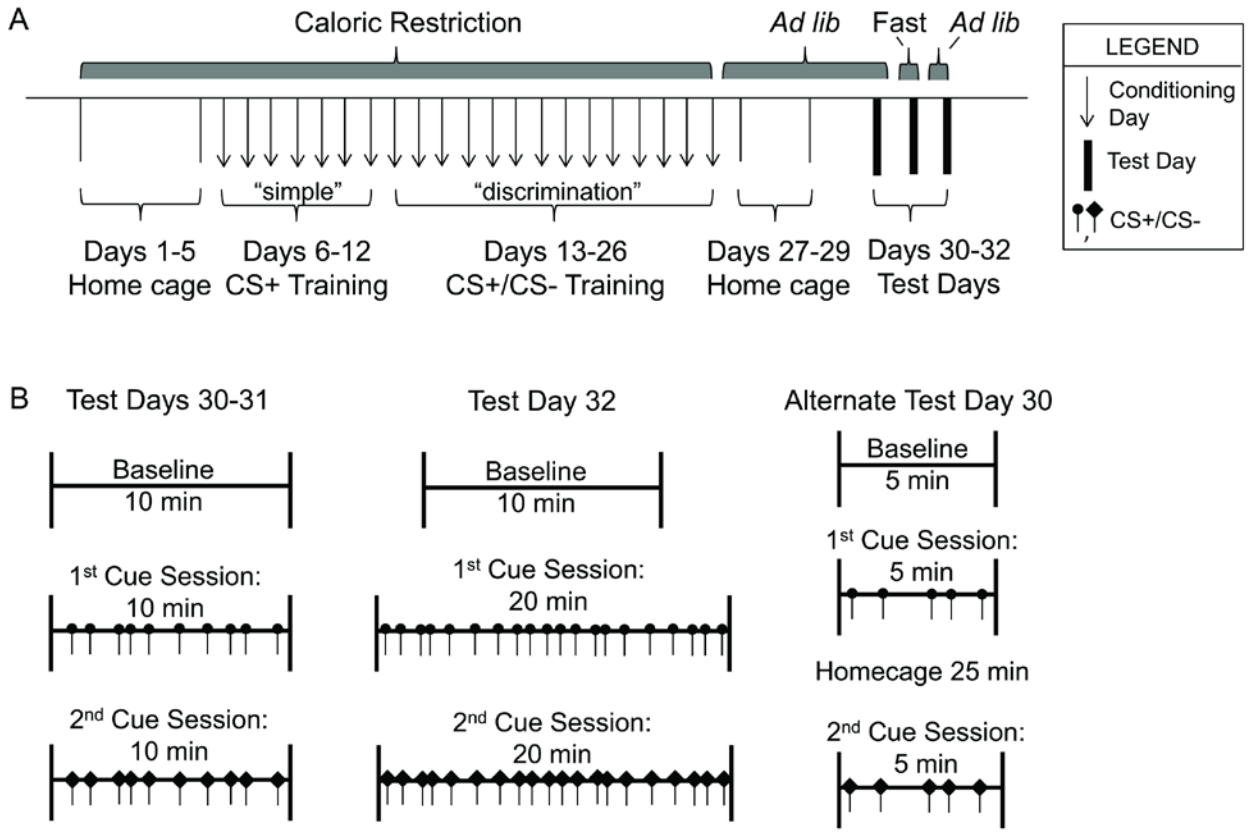

Figure 3.1. Schematic timeline of the primary cue-potentiated feeding protocol adapted for use in mice. A) This protocol consists of a 5 day run-in period during which mice are permitted 3 ½-hr daily access to food in their home cages. While still on this home cage caloric restriction protocol, the mice are exposed to 1 wk of daily "simple" conditioning sessions followed by 2 wks of daily "discrimination" conditioning sessions. For the next 3 days, mice are kept in their home cages with ad lib access to food, after which cue-potentiated feeding responses are tested on Day 30 (Experiments 1 and 4) or Days 30-32 (Experiment 3). B) Test days consist of 3 Test Sessions (Baseline, 1st Cue, and 2nd Cue) in the conditioning apparatus, as indicated.

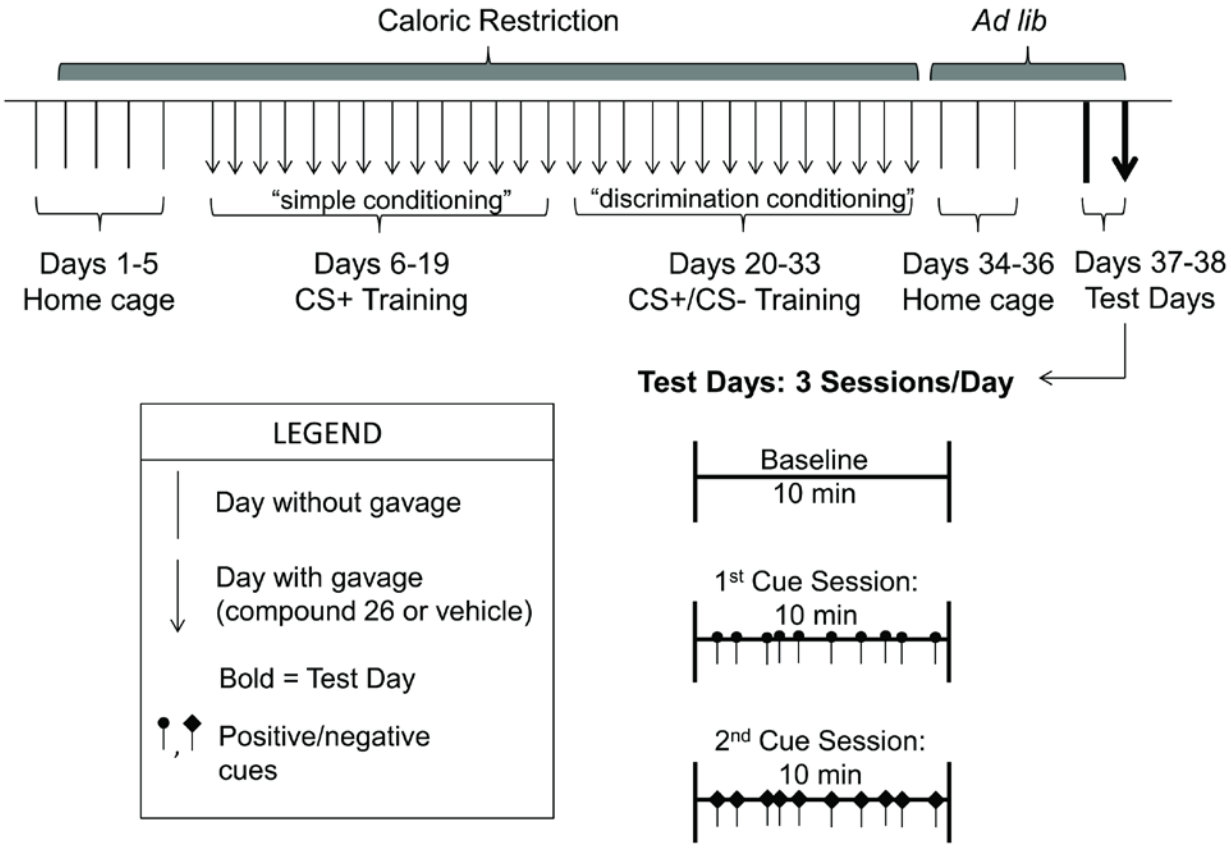

Figure 3.2. Schematic timeline of cue-potentiated feeding protocol modified for use in mice administered GHSR antagonist. Modifications of the primary protocol include the following: a prolonged simple conditioning period (2 wks), use of 20 mg grain-based pellets instead of standard chow during the 3 ½ hour-long daily home-cage feeding period, and 2 test days (1st test day - no gavage, 2nd test day - gavage 1 hr before test).

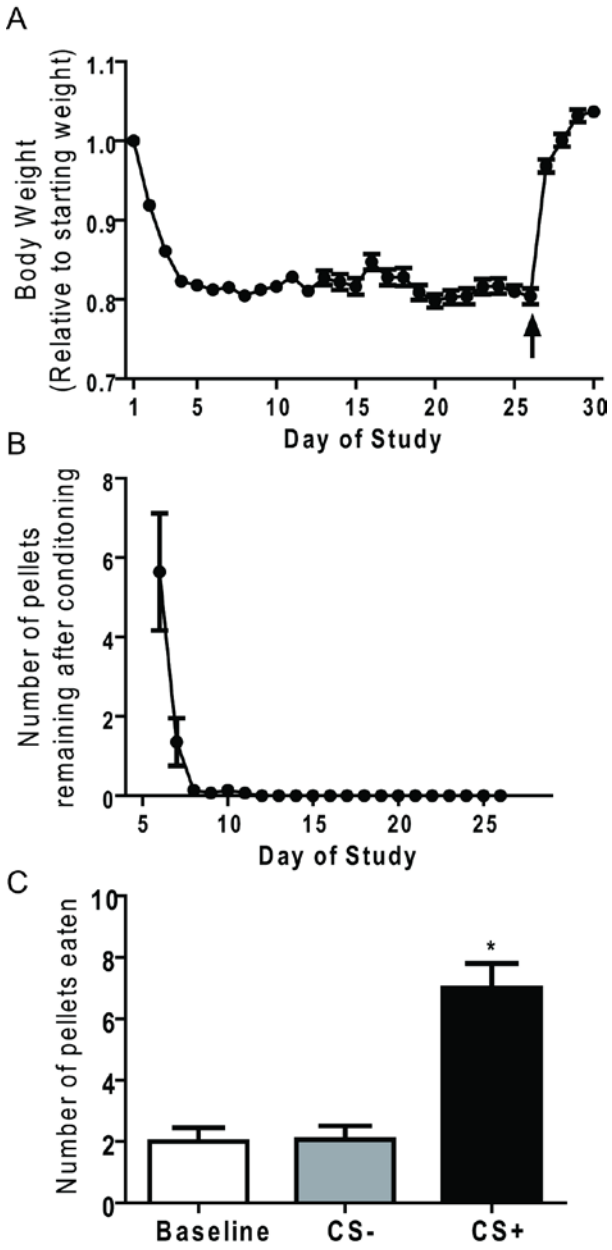

Figure 3.3. Responses of C57BL/6J mice to the cue-potentiated feeding protocol (Experiment 1). A) Body weights of mice throughout the time course of the cue-potentiated feeding protocol (arrow indicates return to ad-lib feeding). B) The number of pellets remaining after each conditioning session. C) Test Day 30 cue-potentiated feeding responses. [n=14; * represents significant difference from other test sessions (*P <0.05)].

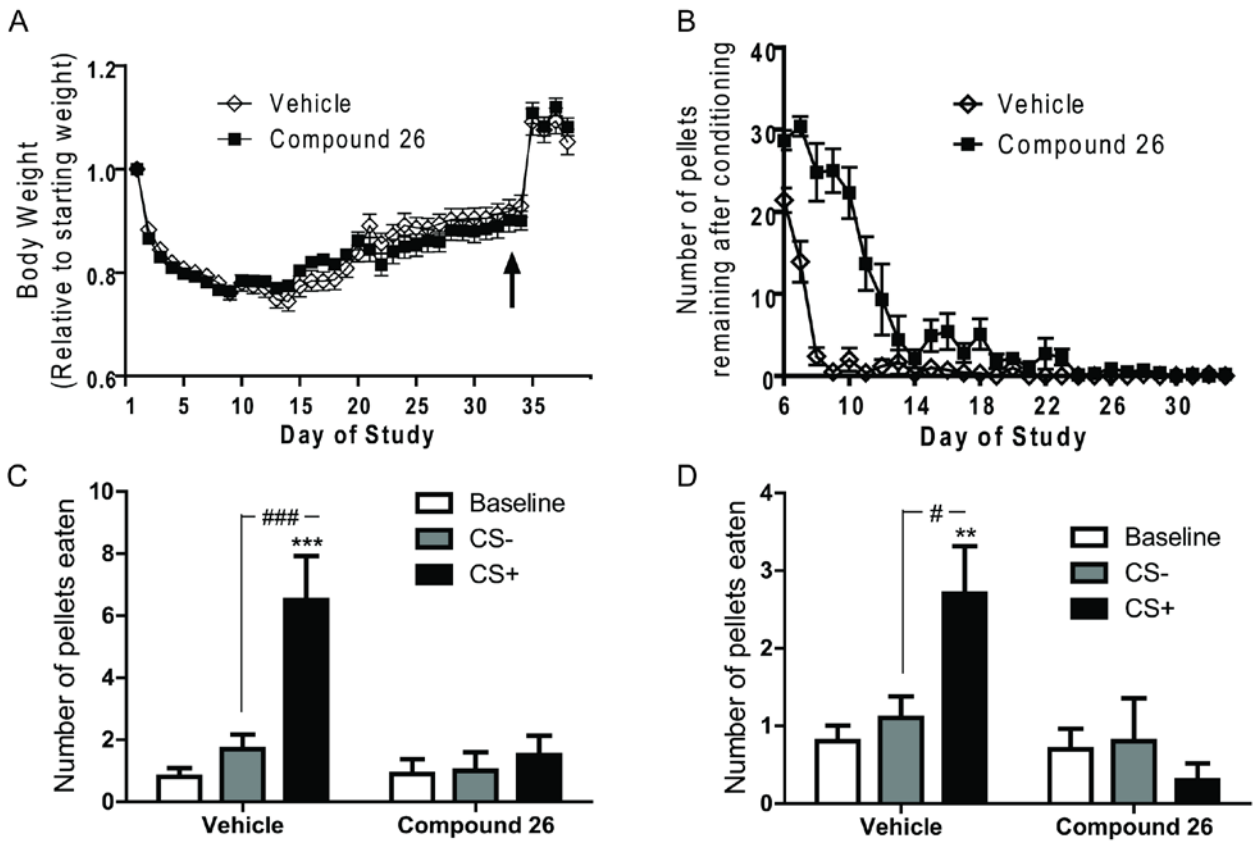

Figure 3.4. Cue-potentiated feeding responses of wild-type mice receiving Compound 26 or vehicle (Experiment 2). A) Body weights of mice throughout the time course of the cue-potentiated feeding protocol, during which either Compound 26 or vehicle was provided 1 hr prior to each conditioning session (arrow indicates return to ad-lib feeding). B) Number of pellets remaining after each conditioning session. C) Test Day 37 feeding responses to positive and negative cues by mice. D) Test Day 38 feeding responses to positive and negative cues by mice given oral gavage of Compound 26 or vehicle 1 hr before testing. [n=10/group; **, *** represent cue sessions significantly different from baseline (**P <0.01, ***P <0.001); #, ### represent cue sessions significantly different from each other (#=P <0.05, ###P <0.001)].

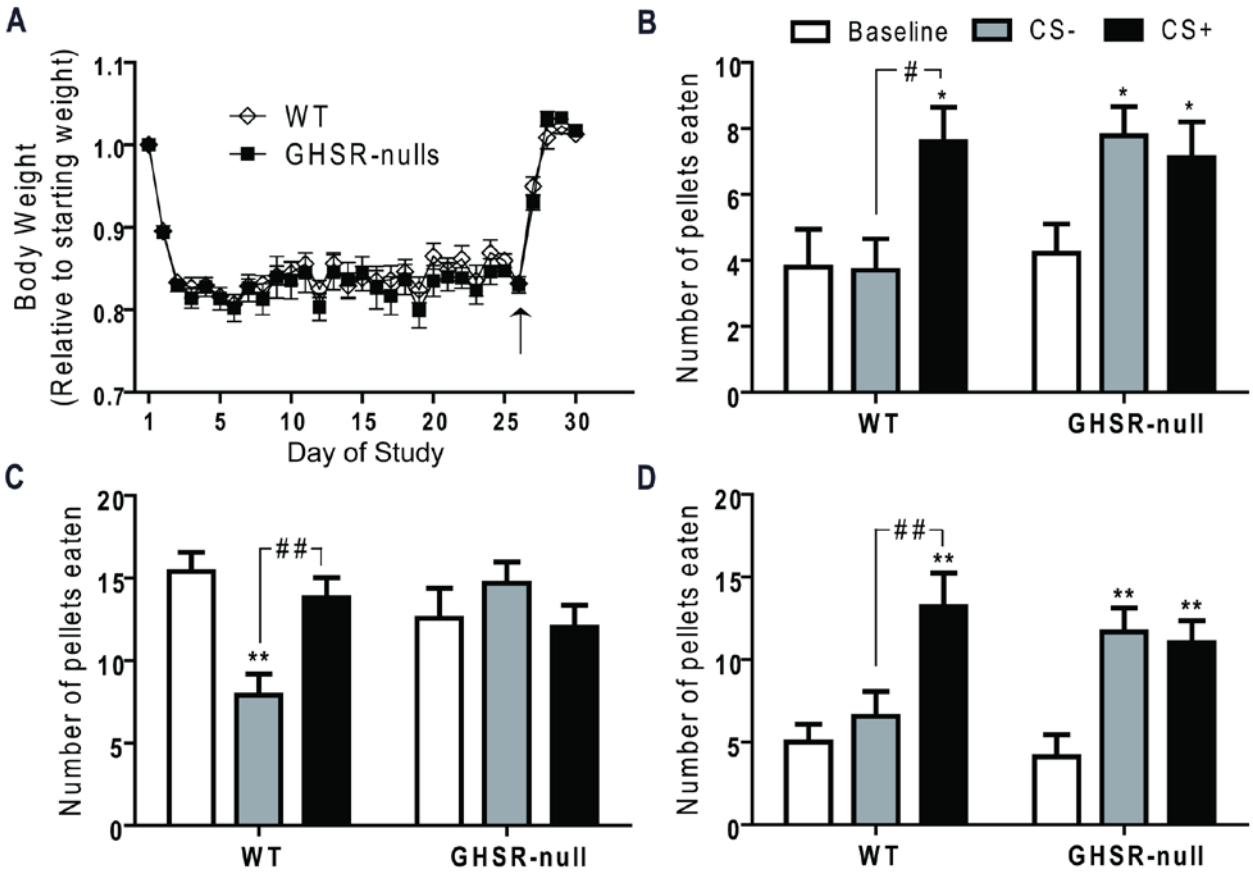

Figure 3.5. Responses of GHSR-null and wild-type littermates to the cue-potentiated feeding protocol (Experiment 3). A) Body weights of mice throughout the time course of the cue-potentiated feeding protocol (arrow indicates return to ad-lib feeding). B) Test Day 30 feeding responses to positive and negative cues by ad lib-fed mice. C) Test Day 31 feeding responses to positive and negative cues by overnight-fasted mice. D) Test Day 32 feeding responses to positive and negative cues by ad lib-fed mice when permitted extended access to the pellets. Legend in Panel B also applies to panels C-D. [n=10/group; *, ** represent cue sessions significantly different from baseline (*P <0.05, **P <0.01); #, ## represent cue sessions significantly different from each other (#=P <0.05, ##P <0.01)].

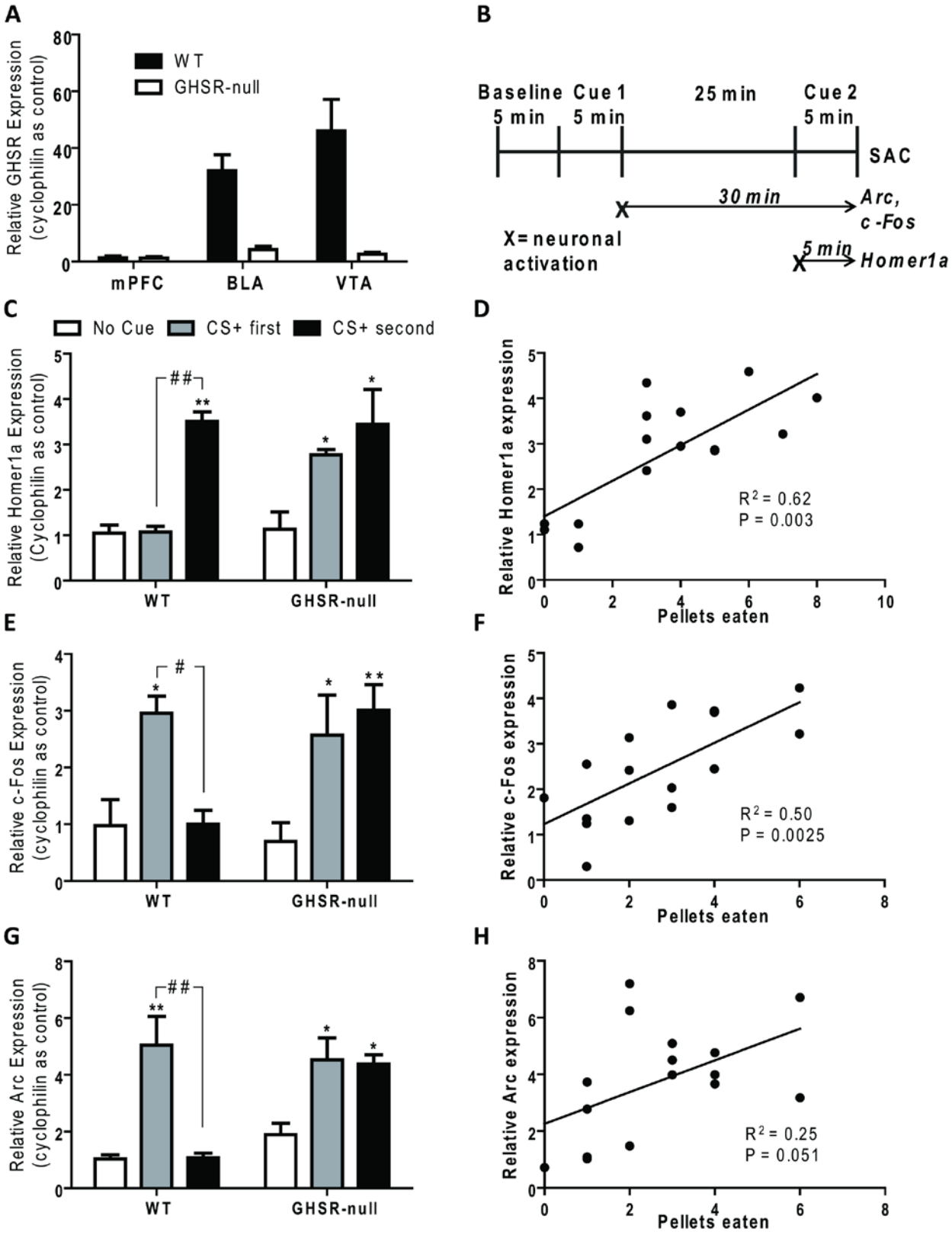
A
Relative GHSR Expression (cyclophilin as control)
WT
GHSR-null
mPFC
BLA
VTA
B
Baseline 5 min
Cue 1 5 min
25 min
Cue 2 5 min
SAC
30 min
Arc, c-Fos
X= neuronal activation
5 min
Homer1a
C
No Cue
CS+ first
CS+ second
Relative Homer1a Expression (Cyclophilin as control)
WT
GHSR-null
D
Relative Homer1a expression
R2 = 0.62
P = 0.003
Pellets eaten
E
Relative c-Fos Expression (cyclophilin as control)
WT
GHSR-null
F
Relative c-Fos expression
R2 = 0.50
P = 0.0025
Pellets eaten
G
Relative Arc Expression (cyclophilin as control)
WT
GHSR-null
H
Relative Arc expression
R2 = 0.25
P = 0.051
Pellets eaten

Figure 3.6. GHSR and immediate early gene expression in the basolateral amygdala.

A) Expression levels of GHSR mRNA in selected brain regions of wild-type and GHSR-null littermates, as determined by qPCR. (n=7/group). B) Schematic illustrating the timing of BLA neuronal activation based on qPCR detection of peak expression levels of mRNA encoding Arc, c-Fos and Homer1a (peak c-Fos and Arc mRNA levels occur at time of sacrifice if neurons activate during first cue test; peak Homer1a mRNA levels occur at time of sacrifice if neurons activate during second cue test). C,E,F) Immediate early gene expression levels, as determined by qPCR, in BLA tissue punches of mice sacrificed immediately following the second cue test. Legend in Panel C also applies to panels E,F. [n=3-4/group; *, ** represent groups significantly different from "no cue" control group (*P <0.05, **P <0.01); #, ## represent groups significantly different from each other (#=P <0.05, ##P <0.01)]. D,F,G) Correlation plots showing relative gene expression as compared to number of pellets eaten for representative test session [n=16]. Each point represents a single mouse including both GHSR-null and wild-type littermates. Correlation coefficients of determination (R^2) are displayed along with the P-values.

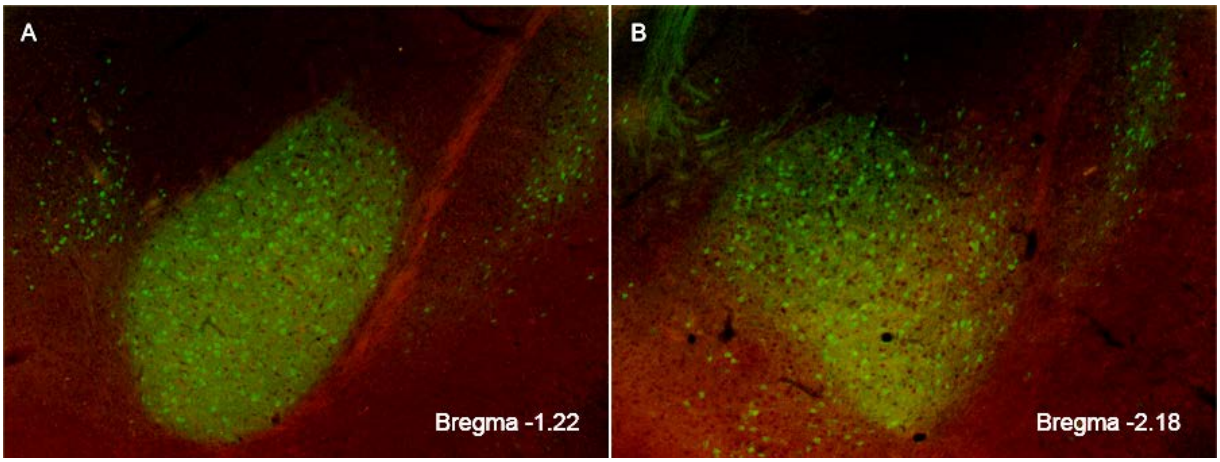

Figure 3.7. eGFP expression in the basolateral amygdala of GHSR-eGFP mice. eGFP expression observed in the BLA of GHSR-eGFP mice at a bregma of -1.22 (A) or -2.18 (B).

CHAPTER FOUR

Expression of Serum Retinol Binding Protein and Transthyretin within Mouse Gastric Ghrelin Cells

Adapted from: **Walker AK**, Gong Z, Park WM, Zigman, JM, Sakata I. Expression of Serum Retinol Binding Protein and Transthyretin within Mouse Gastric Ghrelin Cells. (2013). PLoS One. 8(6):e64882.

Abstract

Ghrelin is an orexigenic peptide hormone produced mainly by a distinct group of dispersed endocrine cells located within the gastric oxyntic mucosa. Besides secreted gene products derived from the preproghrelin gene, which include acyl-ghrelin, desacyl-ghrelin and obestatin, ghrelin cells also synthesize the secreted protein nesfatin-1. The main goal of the current study was to identify other proteins secreted from ghrelin cells. An initial gene chip screen using mRNAs derived from highly enriched pools of mouse gastric ghrelin cells demonstrated high levels of serum retinol-binding protein (RBP4) and transthyretin (TTR), both of which are known to circulate in the bloodstream bound to each other. This high expression was confirmed by quantitative RT-PCR using as template mRNA derived from the enriched gastric ghrelin cell pools and from two ghrelin-producing cell lines (SG-1 and PG-1). RBP4 protein also was shown to be secreted into the culture medium of ghrelin cell lines. Neither acute nor chronic caloric restriction had a significant effect on RBP4 mRNA levels within stomachs of C57BL/6J mice, although both manipulations significantly decreased stomach TTR mRNA levels. In vitro studies using PG-1 cells showed no effect on RBP4 release of octanoic

acid, epinephrine or norepinephrine, all of which are known to act directly on ghrelin cells to stimulate ghrelin secretion. These data provide new insights into ghrelin cell physiology, and given the known functions of RBP4 and TTR, support an emerging role for the ghrelin cell in blood glucose handling and metabolism.

Introduction

The gastrointestinal tract is home to numerous endocrine cell types, the hormonal products of which play critical roles in several physiologic processes and behaviors, including eating, energy homeostasis, glucose metabolism, and gastrointestinal motility (Sanger et al. 2008, Field et al. 2010). Ghrelin is one such gastrointestinal product that is unique in that it is the only known peptide hormone produced in the periphery that potently stimulates food intake (Kojima et al. 1999). Ghrelin is also unique in that it is the only known peptide to be post-translationally modified by *O*-acylation with an octanoic acid group, which is essential for ghrelin binding to its only identified receptor, GHSR (growth hormone secretagogue receptor) (Kojima et al. 1999, Gutierrez et al. 2008, Yang et al. 2008). Besides enhancing food intake, ghrelin also regulates growth hormone secretion, several food reward behaviors, body weight, blood glucose balance, gastrointestinal prokinesis and mood-related behaviors, among many other actions (Tschop et al. 2000, Wren et al. 2000, Asakawa et al. 2001, Nakazato et al. 2001, Dezaki et al. 2004, Zigman et al. 2005, Sun et al. 2006, Lutter et al. 2008, Perello et al. 2010, Zhao et al. 2010, Chuang et al. 2011, Walker et al. 2012).

Ghrelin expression is found throughout the gastrointestinal tract, although most ghrelin circulating in the bloodstream is thought to emanate from a distinct group of sparsely distributed gastric mucosal ghrelin cells (Kojima et al. 1999, Date et al. 2000, Dornonville de la Cour et al.

2001, Sakata et al. 2002). Prior to the discovery of ghrelin, these cells had been identifiable due to their characteristic round, compact, electron-dense secretory granules (Date et al. 2000, Dornonville de la Cour et al. 2001, Rindi et al. 2002, Yabuki et al. 2004). Using mRNA derived from enriched pools of ghrelin cells [isolated from transgenic mice which express humanized *Renilla reniformis* green fluorescent protein (hrGFP) under the control of the ghrelin promoter], the expression of chromogranin A and prohormone convertases 1/3 and 2 within gastric ghrelin cells has previously been described, thus confirming the peptide hormone-producing nature of these cells (Sakata et al. 2009). Similar types of quantitative RT-PCR (qPCR) analyses on fluorescence activated cell sorting (FACS)-separated, GFP-labeled ghrelin cells have been used to determine the membrane-bound *O*-acyltransferase and adrenergic receptor expression profile of ghrelin cells, as well as their expression of several enzymes, receptors, and channels that potentially help mediate the response of ghrelin cells to D-glucose and other hormones (Sakata et al. 2009, Zhao et al. 2010, Lu et al. 2012, Sakata et al. 2012). A combination of qPCR analyses and cell culture techniques using immortalized ghrelin cell lines derived from transgenic mice containing gastric ghrelinomas has helped establish the β_1-adrenergic receptor system as a key regulator of ghrelin biosynthesis and secretion (Zhao et al. 2010, Iwakura et al. 2011). Ghrelin secretion in response to direct exposure of immortalized gastric ghrelin cell lines to a variety of other peptide hormones, monoamines, and other compounds also has been investigated, with a resulting inhibitory or null effect described for insulin and somatostatin and a stimulatory effect identified for dopamine and oxytocin (Iwakura et al. 2010, Zhao et al. 2010, Iwakura et al. 2011). Many of these findings have been reproduced in ghrelin cell-containing primary cultures of dispersed mouse or rat gastric mucosal cells and in primary cultures of highly purified pools of gastric ghrelin cells (Lu et al. 2012, Sakata et al. 2012, Gagnon et al. 2013, Gagnon et al. 2013). Much remains to be discovered regarding ghrelin cell physiology and pathophysiology.

Accumulating evidence suggests that gastric ghrelin cells synthesize more than just one secreted product (Stengel et al. 2012). Both acyl- and desacyl-forms of ghrelin are presumed to be released from ghrelin cells (Hosoda et al. 2000, Mizutani et al. 2009, Zhao et al. 2010, Sakata et al. 2012). In addition, and similar to enteroendocrine L-cells which secrete multiple peptide hormones resulting from prohormone convertase-mediated cleavage of the preproglucagon gene, ghrelin cells secrete at least two gene products derived from prohormone convertase 1/3-mediated cleavage of the preproghrelin gene: ghrelin and obestatin (Rouille et al. 1995, Zhang et al. 2005, Zhu et al. 2006). Obestatin is a 23-amino acid peptide that was originally reported to have effects on food intake and gastrointestinal motility opposite to those of acyl-ghrelin, although these findings have not been replicated in all published reports (Zhang et al. 2005, Seoane et al. 2006, Gourcerol et al. 2007, Nogueiras et al. 2007, Kobelt et al. 2008, Stengel et al. 2010). Parenthetically, while the orexigenic effects of acyl-ghrelin are well-established, the biological effects of desacyl-ghrelin – particularly its effects on food intake – have been mixed, ranging from decreased to absent to increased (Asakawa et al. 2005, Neary et al. 2006, Toshinai et al. 2006). Nesfatin-1 is another peptide hormone that has been found to be highly co-localized with ghrelin within the stomach (Stengel et al. 2009). Interestingly, nesfatin-1, which has appetite suppressing actions as well as stimulatory effects on glucagon secretion, also results from prohormone convertase 1/3-mediated processing of its precursor (nucleobindin 2), and is regulated oppositely from ghrelin at the mRNA level in the setting of caloric restriction (Toshinai et al. 2001, Stengel et al. 2009, Stengel et al. 2010, Riva et al. 2011). The presence of prohormone convertase 2 within gastric ghrelin cells, which unlike prohormone convertase 1/3 is not involved in the processing of proghrelin to ghrelin(Ozawa et al. 2007, Sakata et al. 2009, Lu et al. 2012), suggests that other peptide hormones may be present in ghrelin cells.

The current study sought to identify and further characterize other presumed metabolically-relevant proteins secreted by gastric ghrelin cells. These investigations identified high ghrelin cell expression of both retinol-binding protein (RBP4) and transthyretin (TTR). RBP4 not only transports retinol in the bloodstream, but also has been linked to insulin resistance in both rodent and human models (Yang et al. 2005, Graham et al. 2006). Transthyretin, previously known as prealbumin and subsequently thyroxine-binding prealbumin, is so named because of its function in transporting both thyroid hormone and retinol binding protein in the bloodstream (Richardson 2009). Other explorations included ghrelin cell-specific secretion and transcriptional regulation of RBP4 and TTR upon caloric restriction and exposure to octanoic acid and β_1-adrenergic receptor agonists.

Materials and Methods

Ethics Statement

All studies were approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center (Animal Protocol Numbers 2009-0377 and 2008-0107) and in accordance with the Saitama University Committee on Animal Research.

Animals

Tissues (stomach, perigonadal white adipose tissue and liver) for quantitative RT-PCR (qPCR) were from 12 wk-old male C57BL/6J mice housed with *ad libitum* access to water and standard rodent diet (Diet 7001; Harlan Teklad, Madison, WI) in a light- and temperature-controlled facility. For certain of the qPCR studies, stomach samples were removed from 10 wk-

old male C57BL/6J mice following a 24-hr fast or following 7-days of 60% caloric restriction. For the 60% caloric restriction studies, daily food intake was assessed for each individually-housed study animal during a 3-day run-in period, after which mice were given daily access to only 60% of their usual (average) daily intake. Food restricted mice maintained *ad libitum* access to water. To obtain RNA from ghrelin cell-enriched pools of gastric mucosa cells, 12 wk-old male transgenic ghrelin-hrGFP (humanized *Renilla reniformis* green fluorescent protein) reporter mice (line hrGFP10) were used (Sakata et al. 2009). For measurement of plasma RBP4, 10 wk-old and 20 wk-old male transgenic mice harboring ghrelinomas (TgGhrelin-SV40-T) were used (Zhao et al. 2010). For blood collection, mice were deeply anesthetized with an intraperitoneal injection of chloral hydrate (500 mg/kg body weight), after which blood was collected from the inferior vena cava in EDTA-coated tubes containing *p*-hydroxymercuribenzoic acid (final concentration, 1 mM; Sigma-Aldrich, St. Louis, MO). The plasma was separated immediately, acidified with 1N HCl to a final concentration of 0.1 N HCl, and then stored at −80°C until analysis.

Isolation of ghrelin cell-enriched and non-ghrelin cell-enriched pools of gastric mucosal cells using ghrelin-hrGFP transgenic mice

Separate pools of gastric mucosal cells that were either enriched for ghrelin cells (hrGFP/ghrelin-cell enriched pools) or that mostly lacked ghrelin cells (hrGFP-negative pools) were isolated from transgenic ghrelin-hrGFP mice (line hrGFP10). This process involved a gentle mechanical and enzymatic release of gastric mucosal cells from the stomach followed by cell sorting (MoFlo, Dakocytomation, USA) at the UTSW Medical Center Flow Cytometry Multi-user Core Facility, as described in more detail previously (Sakata et al. 2009, Sakata et al. 2009, Zhao et al. 2010, Sakata et al. 2012). Four independent fluorescence activated cell

sorting (FACS) preparations were included in the subsequent analyses, with 3–5 mice being used for each independent FACS preparation.

RNA extraction and quantitative RT-PCR

C57BL/6J tissues for qPCR were collected immediately after mice were sacrificed, and were quickly immersed in RNAlater reagent (Qiagen, Hilden, Germany) and then stored at −20°C until extraction. Total RNA was extracted from these tissues using STAT60 (Arcturus Bioscience, Mountain View, CA), according to the manufacturer's protocol. After FACS sorting of hrGFP10 gastric mucosal cells, the hrGFP/ghrelin-cell enriched pools and the hrGFP-negative pools were adjusted to contain a matched number of cells, and the cells in each pool were collected by centrifugation at 4°C at 3000 rpm, for 10 min. Total RNA was extracted from these cellular pellets using STAT60, and was stored at −80°C until use. Total RNA was also extracted from two different mouse ghrelinoma cell lines, SG-1 and PG-1(Zhao et al. 2010). Complementary DNA was synthesized from 100 ng total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The following primer pairs were designed to amplify ghrelin: *5′-GTC CTC ACC ACC AAG ACC AT-3′* and *5′-TGG CTT CTT GGA TTC CTT TC-3′*; RBP4: *5′-ACT GGG GTG TAG CCT CCT TT-3′* and *5′-GGA GTA CTG CAG AGC GAA GG-3′*; transthyretin: *5′-TGG ACA CCA AAT CGT ACT GG-3′* and *5′-GAT GGT GTA GTG GCG ATG G-3′*; nucleobindin 2: *5′-AAA AGC TCC AGA AAG CAG ACA-3′* and *5′-GCT CAT CCA GTC TCG TCC TC-3′*; cyclophilin: *5′-TGG AGA GCA CCA AGA CAG ACA-3′* and *5′-TGC CGG AGT CGA CAA TGA T-3′*. Messenger RNA levels were measured with an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster, City, CA). Quantitative RT-PCR was performed using the iTaq SYBR Supermix (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Initial template denaturation (3 min at 95°C) was performed, followed by 40 cycles

of denaturation (15 sec at 95°C) and annealing/extension (45 sec at 60°C). Reactions were evaluated by the comparative threshold cycle (C_T) method, using cyclophilin as the invariant control gene. The previously reported C_T values of several genes amplified from FACS-separated gastric mucosal cells compared with the C_T values of a separate housekeeping gene, 36B4, revealed results similar to those determined with cyclophilin (Sakata et al. 2009). The efficiencies of the primers were validated by verifying a slope of approximately −3.3 when the logs of the cDNA concentration at different serial dilutions were compared with the C_T. The primers were designed to amplify regions of cDNA that in the corresponding genomic DNA span introns to ensure the amplification of cDNA derived from mRNA rather than residual genomic DNA.

Gene chip array analysis

After FACS separation of hrGFP10 gastric mucosal cells, the hrGFP/ghrelin-cell enriched pools and the hrGFP-negative pools were adjusted to contain a matched number of cells (15,000 cells), and the cells in each pool were collected by centrifugation at 4°C at 3000 rpm, for 10 min. Total RNA was extracted from the cells using the Pico Pure RNA isolation kit (Arcturus Bioscience). Fifty ng total RNA was used for making labeled cRNA with the Affymetrix Two-Cycle Target Labeling Protocol (Affymetrix, Santa Clara, CA). Labeled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Hybridization, washing and scanning were carried out by the UTSW Microarray Core Facility. Data were analyzed with GeneChip Operating Software version 1.0 using the mas5 algorithm.

In vitro secretion studies

Cells from the immortalized ghrelin cell line PG-1 were used for these studies (Zhao et al. 2010). On day 0, PG-1 cells were plated in DMEM/F-12 50:50 medium (Mediatech, Inc., Manassas, VA) containing 10% (vol/vol) fetal bovine serum (FBS) and supplemented with 50 µM octanoate-BSA, 100 U/mL penicillin and100 µg/mL streptomycin sulfate. The cells were placed into the wells of poly-L-lysine-coated 24-well plate culture dishes and incubated in humidified 95% air and 5% CO_2 at 37°C. Cells were maintained in DMEM/F-12 with 10% FBS in a 37°C incubator with 5% CO_2. On day 2, the media was aspirated and replaced with serum-free DMEM containing 5.5 mM glucose, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate with different concentrations of octanoate-BSA. Alternatively on day 2, the media was aspirated and replaced with serum-free DMEM containing 5.5 mM glucose, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate and 50 µM octanoate-BSA containing either 10 µM epinephrine or 10 µM norepinephrine (Sigma-Aldrich). The day 2 incubations were performed for 6 hours in humidified 95% air and 5% CO_2 at 37°C, and then the media was immediately collected and centrifuged at 3,000× g for 5 min. The supernatant was stored at −80°C until analysis. These culture conditions are slightly different than those previously reported, however these slight modifications did not alter the previously-reported ghrelin secretory response of the cells to norepinephrine (Zhao et al. 2010).

Detection of RBP4 protein by ELISA and Western Blot analysis

RBP4 protein levels were determined using RBP4 (mouse/rat) Dual ELISA kit (Cat. No. AG-45A-0012EK-KI01, Adipogen, Incheon, Korea), according to the manufacturer's instructions. In addition, the presence of RBP4 protein was determined using Western Blot analysis. To

prepare samples, SG-1 cells were cultured with DMEM/F-12 with 10% FBS in a 37°C incubator with 5% CO_2 and 5% O_2 for 2 days, after which the cells and culture media were collected together and centrifuged at 1200× rpm for 5 min. The supernatant (culture media) was separated from the cell pellet and stored at −80°C until protein measurement. The cell pellet was washed twice with cold PBS, after which 1 mL RIPA buffer (Nacalai Tesque, Inc., Kyoto, Japan) including 1× PMSF (Sigma) and 1× protease inhibitor cocktails (Nacalai Tesque) was added. The cells were homogenized for 10 min while on ice, and the resulting cell lysate was centrifuged at 12,000× g for 10 min. The supernatant (cell lysate) was stored at −80°C until protein measurement. Total protein concentrations of the culture media and cell lysate samples were measured by BCA™ Protein Assay kit (Thermo, Rockford, USA) according to the manufacturer's instructions. Equal amounts of protein (45 µg/well for cell lysate, 17 µg/well for medium) were run on 12% sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto PVDF membranes. The membranes were probed successively with primary anti-human Retinol-Binding Protein antisera (Lot:00066392, Dako, Glostrup, Denmark) diluted 1:1000 and then HRP-labeled secondary antibody (Promega, Madison, WI, USA) diluted in 1:100,000, for 2 h at room temperature. Specific antigen-antibody binding was visualized with LuminataTM Forta Western HRP substrate (Millipore Corporation, Billerica, USA) using a ChemiDoc XRS System (Bio Rad, Hercules, CA, USA).

Ghrelin measurements

Acyl-ghrelin concentrations were determined using a Rat acylated ghrelin enzyme immunoassay kit (#A05117, Bertin pharma, Montigny le Bretonneux, France) according to the manufacturer's guidelines. Samples were diluted 1: 5 for the measurements.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses. For Table 4.2 qPCR studies, one-way ANOVA followed by Dunnett's post hoc test was used to compare levels of mRNA in each tissue to whole stomach; in cases of high variance, log transformations of data were performed prior to statictical analysis. For all other analyses, one-way ANOVA followed by Tukey's post hoc test was used and $P<0.05$ was considered to be statistically significant.

Results

Expression of RBP4 and transthyretin (TTR) mRNA in ghrelin cells

In an effort to broadly identify proteins expressed highly within ghrelin cells, a gene chip array was screened that had been prepared from RNA isolated from pools of gastric mucosal cells highly enriched for ghrelin cells (hrGFP/ghrelin-cell enriched pools). Data from this gene chip were compared to those generated using RNA isolated from pools of gastric mucosal cells from which ghrelin cells had been mostly removed (hrGFP-negative pools). Separation of the different gastric mucosal pools was achieved by fluorescence activated cell sorting of gastric mucosal cells isolated from transgenic ghrelin-hrGFP reporter mice, as done previously (Sakata et al. 2009, Sakata et al. 2009, Zhao et al. 2010, Sakata et al. 2012). A selected list of molecules identified using this method is included in Table 4.1. As expected, signal intensities reflecting numbers of ghrelin and ghrelin O-acyltransferase (GOAT) mRNA transcripts, were markedly higher on the gene chip probed with mRNA from hrGFP/ghrelin cell-enriched pools as compared to the gene chip probed with mRNA from hrGFP-negative pools. Messenger RNA for serum retinol binding protein 4 (RBP4), which is of interest due to the known association of its

encoded protein with disorded metabolic states, also was present at relatively high levels in the hrGFP/ghrelin cell-enriched pools. Because RBP4 is known to circulate in the bloodstream bound to transthyretin (TTR), the gene chip was also searched for TTR mRNA and its signal intensity was found to be increased in the hrGFP/ghrelin cell-enriched pools. For comparison, chromogranin A, prohormone convertase 1/3, and prohormone convertase 2 mRNA expression had been previously identified within ghrelin cells (Sakata et al. 2009), and these again were identified as being present at relatively high levels in the hrGFP/ghrelin cell-enriched pools. As expected from previous ghrelin co-localization studies, nucleobindin 2 (which encodes nesfatin-1) and regulated endocrine-specific protein 18 (RESP18), an endoplasmic reticulum-localized protein of unclear physiological function, also both were found at high levels in the hrGFP/ghrelin cell-enriched pools (Stengel et al. 2009, Erlandsen et al. 2012).

Relative levels of selected set of mRNAs within FACS-separated pools of gastric mucosal cells, as determined by Affymetrix array.

To independently confirm the expression data revealed by the gene chip arrays, quantitative RT-PCR analysis on several tissue samples was performed using specific oligonucelotide primers (Table 4.2). Amplification of complementary DNA prepared from whole stomach and FACS-separated gastric mucosal cells from ghrelin-hrGFP mouse stomachs demonstrated significantly higher ghrelin mRNA levels in the hrGFP/ghrelin cell-enriched pools and in two different immortalized ghrelin cell lines derived from transgenic mice harboring SV40 large T-antigen-induced ghrelinomas (SG-1 and PG-1) as compared to whole stomach, as demonstrated previously (Zhao et al. 2010). Similarly, as compared to their mRNA expression levels within whole stomach, RBP4 and TTR levels within highly enriched pools of FACS-separated gastric ghrelin cells were substantial, as were RBP4 and TTR levels within the

ghrelinoma cell lines. As had been observed previously for ghrelin transcripts and other transcripts within SG-1 and PG-1 cell lines, levels of RBP4 and TTR transcripts within the ghrleinoma cell lines did not match exactly those observed in the FACS-separated gastric ghrelin cells(Zhao et al. 2010, Sakata et al. 2012). In contrast, ghrelin mRNA was barely detected by qPCR in liver and white adipose tissue, while, as expected from previous work, RBP4 mRNA was detected in both liver and white adipose tissue, and TTR mRNA was high in liver but not white adiopose tissue (Blaner 1989, Richardson 2009). The mean RBP4 mRNA level in the hrGFP/ghrelin cell-enriched pool approached 31% of that in liver and was nearly 95% of that observed in WAT. The mean TTR mRNA within the hrGFP-positive pool was even closer (62%) to that within the liver.

Expression of nucleobindin 2 mRNA in ghrelin cells

Previous work has demonstrated expression of nucleobindin 2 (NUCB2) mRNA and one of its gene products, nesfatin-1, in ghrelin cells (Stengel et al. 2009). Thus, for comparison, NUCB2 mRNA levels were also analyzed using the same qPCR strategy as above. Here, NUCB2 mRNA was detected by qPCR within the hrGFP/ghrelin cell-enriched pools, although levels were not significantly higher than in whole stomach (Table 4.2). Presumably this lack of enrichment is due, at least in part, to expression of NUCB2 in other gastric mucosal cells such as histamine-containing and somatostatin-containing cells in which it also was previously identified (Stengel et al. 2009). As compared to RBP4 and TTR, the relative amounts of NUCB2 mRNA within the hrGFP/ghrelin cell-enriched pools were lower.

Regulation of RBP4 and TTR mRNA expression in whole stomach by caloric restriction

As ghrelin transcription within and secretion from ghrelin cells is known to be regulated by changes in metabolic state, changes in RBP4 and TTR mRNA expression within the whole stomach upon caloric restriction were next examined using a more acute model (24-hour fast) and a more chronic model (daily exposure to only 60% of usual daily calories, for 7 days). In neither the acute nor chronic caloric restriction model were statistically significant changes to RBP4 mRNA observed (Fig. 4.1A). On the other hand, TTR mRNA expression within the stomach was significantly decreased upon a 24-hour fast and 60% calorie restriction, to nearly 70% and 54% the levels observed in stomachs from *ad lib*-fed mice, respectively (Fig. 4.1B).

RBP4 secretion from immortalized ghrelin cell lines

Next, secretion of RBP4 from the PG-1 immortalized ghrelin cell line was examined (Zhao et al. 2010). Previously, both this PG-1 line and a second ghrelinoma cell line (SG-1) were shown to serve as good models with which to study mechanisms of ghrelin release, demonstrating similar gene expression profiles as well as similar responses to candidate ghrelin secretagogues (Zhao et al. 2010). In particular, in prior work, exposures of PG-1 cells and SG-1 cells to octanoic acid, epinephrine and norepinephrine were each shown to dose-dependently enhance secretion of acyl-ghrelin; epinephrine and norepinephrine also stimulated desacyl-ghrelin release (Zhao et al. 2010). Here, although RBP4 was detected by ELISA assay in the culture media of PG-1 cells, neither octanoic acid, using doses ranging from 0.1 µM to 100 µM, 10 µM epinephrine nor 10 µM norepinephrine, altered levels of RBP4 in the media upon a 6-hour exposure (Fig. 4.2)

Similarly, RBP4 protein was detected in both SG-1 cell culture media and SG-1 cell lysates by Western blot analysis (Fig. 4.3). However, neither a similar range of octanoic acid doses nor 10 µM norepinephrine was able to stimulate RBP4 release from SG-1 cells, despite the ability of the 10 µM norepinephrine to stimulate ghrelin release into the culture media, as had been demonstrated previously (Zhao et al. 2010) (data not shown). Lack of a similar pattern of stimulated RBP4 and ghrelin secretion by octanoic acid and catecholamines suggests that RBP4 and ghrelin cellular localization and secretion regulatory mechanisms also are not similar.

Plasma RBP4 levels in mice harboring ghrelinomas

Next, a study was performed to determine if expanded ghrelin cell numbers could influence circulating levels of RBP4 *in vivo*. To accomplish this, plasma RBP4 levels were measured using the same transgenic mouse line used to derive the PG-1 and SG-1 ghrelinoma cell lines, in transgenic mice and wild-type littermates aged 10 wks and 20 wks. Previously, it had been demonstrated that there were approximately 2-fold increases in acyl-ghrelin and desacyl-ghrelin plasma levels in 10 wk-old mice harboring the ghrelin-SV40 T-antigen transgene as compared to 10 wk-old wild-type littermates; a 25-fold increase in plasma acyl-ghrelin and a 37-fold increase in plasma desacyl-ghrelin were noted in 20 wk-old transgenic mice (Zhao et al. 2010). The marked increase in ghrelin in the 20 wk-old mice has been presumed to correspond with the SV40 T-antigen-driven clonal expansion of ghrelin cells (Zhao et al. 2010). Of note, for most of the mice harboring the ghrelin-SV40 T-antigen transgene, noticeable abdominal cavity tumors do not become apparent in live animals until the age of 24 wks, although the elevated plasma ghrelin in younger mice suggests the presence of hyperplastic ghrelin cells at those earlier ages (Zhao et al. 2010). Here, despite the expanded mass of ghrelin cells, no statistically

significant increase in plasma RBP4 levels was apparent in either 10 wk-old mice or 20 wk-old mice (Fig. 4.4).

Discussion

In this paper, the marked expression of the secreted proteins RBP4 and TTR is described for within gastric ghrelin cells for the first time. For RBP4, this marked expression was first suggested at the mRNA level in FACS-sorted hrGFP-positive cells from ghrelin-hrGFP reporter mice, using gene chip technology. Such was confirmed by qPCR studies using not only the hrGFP/ghrelin cell-enriched samples but also two different mouse ghrelinoma cell lines. Similar to the observed enrichment of RBP4 noted in FACS-sorted hrGFP-positive cells, mRNA for TTR, which serves as a transport protein for both RBP4 and thyroid hormone, also was expressed within the stomach, where it was highly enriched within the ghrelin cell population. Although RBP4 was found in lysates and the media of cultured ghrelinoma cells, octanoic acid or catecholamine stimulation of RBP4 secretion was not observed, as had been observed previously when the same conditions were used to assess acyl-ghrelin secretion (Zhao et al. 2010). Nor were changes observed in RBP4 mRNA levels in whole stomach upon caloric restriction. In addition, there was a lack of statistically significant increases in plasma RBP4 in mice harboring ghrelinomas. Caloric restriction did, however, decrease TTR mRNA in whole stomach.

The significance of these findings stems not only from the known biological functions of RBP4 and TTR, but also from the mostly uncharacterized physiology of the ghrelin cell. RBP4 is a 21-kDa protein synthesized by and secreted mainly from the liver (Blaner 1989). Its best characterized function is to deliver retinol (vitamin A) from hepatic retinoid stores to tissues

throughout the body, where it is metabolized to retinaldehyde and retinoic acid (D'Ambrosio et al. 2011). More specifically, RBP4 is the only specific serum transport protein for retinol and thus serves an important function for a multitude of retinol-dependent functions, including vision, growth and development and immune regulation (Graham et al. 2007, D'Ambrosio et al. 2011).

More recently, plasma RBP4 has been identified as an important signal of insulin resistance (Graham et al. 2007). This first became evident in insulin resistant mice lacking GLUT4 (glucose transporter 4) specifically in white adipose tissue, in which serum RBP4 was markedly elevated as was the level of white adipose tissue RBP4 mRNA (Yang et al. 2005). Previously in rodents, adipose tissue had been reported to contain relatively high levels of RPB4 mRNA, averaging between 6–37% those in liver (Makover et al. 1989, Tsutsumi et al. 1992). Circulating RBP4 is also elevated in several other mouse models of insulin resistance as well as in insulin-resistant humans (Yang et al. 2005, Graham et al. 2006). Interestingly, pharmacologic administration of RBP4 and transgenic overexpression of RBP4 both induce insulin resistance in mice, while genetic deletion of RBP4 enhances insulin sensitivity (Yang et al. 2005). These findings and others suggest that elevated serum RBP4 may play a causative role in the development of insulin resistance, although it is as yet unclear if this involves retinol-dependent mechanisms, retinol-independent mechanisms, or both (Graham et al. 2007).

Retinol-RBP4 circulates in the bloodstream in a 1:1 molar complex with TTR (Episkopou et al. 1993, Quadro et al. 2004). Like RBP4, TTR also is synthesized mainly in the liver (Richardson 2009). Not only does TTR transport the retinol-RBP4 complex in the bloodstream, but also it prevents glomerular filtration of RBP4 at the kidney, thus helping to prevent loss of retinol and RBP4 in the urine (van Bennekum et al. 2001). Regarding its role in thyroid hormone transport in the bloodstream, in humans, TTR carries about 10–15% of T_4 and T_3 (as opposed to thyroxine binding globulin, which carries about 70%, albumin, which carries about 15–20%, and lipoproteins, which carry a fraction), although its relatively low affinity for T4 and T3 leads to

a more immediate delivery of thyroid hormone to target tissues (Robbins et al. 2000). In mice, TTR serves as the major thyroid hormone transport protein (Robbins et al. 2000). TTR also is synthesized in the choroid plexus, which likely accounts for its presence in cerebrospinal fluid (Robbins et al. 2000). Within the CSF of both humans and mice, TTR functions as the major carrier of thyroid hormone; in this role, TTR is thought to facilitate the passage of thyroid hormone across the choroid plexus and then distribute it to the central nervous system (Robbins et al. 2000).

Of interest, RBP4 and TTR expression have been localized to more sites than just the liver plus white adipose tissue (RBP4) and the liver plus choroid plexus (TTR). Using Northern Blot analysis on various rat tissues, RBP4 mRNA has been detected in the kidney at 5–10% of hepatic levels, in lung, spleen, brain, heart and skeletal muscle at 1–3% of hepatic levels, and in the intestine, testis and pancreas at <1% of hepatic levels (Soprano et al. 1988). Other studies have shown RBP mRNA in rat eye, where it is localized to the retinal pigment epithelium, and in the visceral yolk sac (Martone et al. 1988, Soprano et al. 1988). As it relates to the current study, Northern blot analysis also has revealed RBP4 mRNA levels in stomach at 1–3% of hepatic levels (Soprano et al. 1988). Here, using qPCR, RBP4 mRNA levels within whole stomach reached only 0.4% of those in the liver while RBP4 mRNA levels within the hrGFP/ghrelin cell-enriched pools reached 31% of those in the liver. Thus, the current data suggest that the RBP4 expression within ghrelin cells likely accounts for the majority of its stomach expression. Like RBP4, TTR is also synthesized in the rat pigment epithelium (Cavallaro et al. 1990). Additionally, it is produced in the placenta and in the islets of Langerhans (Kato et al. 1985, Jacobsson 1989). TTR expression within the gastrointestinal tract also has been described previously, although never before co-localized with ghrelin as described in the current study. In particular, transgenic mice in which Cre recombinase or red fluorescence protein expression are directed by TTR promoter elements manifest reporter

activity in the gastric mucosa of E12.5–E16.5 embryonic animals (Kwon et al. 2009). Here, in adult mice, TTR expression in the stomach approached 0.4% of its levels in liver, while that in hrGFP/ghrelin cell-enriched pools approached 62% of its levels in liver.

It has been proposed that the sites of RBP4 and TTR biosynthesis may help dictate their functions. In a study specifically designed to investigate the physiological role of circulating RBP4 produced outside the liver, handling of retinol was examined in mice in which endogenous RBP4 expression was genetically deleted and replaced with RBP4 overexpressed in muscle. Unlike liver-derived RBP4, the extrahepatically synthesized RBP4 failed to mobilize liver retinol stores in this mouse model (Quadro et al. 2004). This led the authors of that study to postulate that RBP4 "has a function specific to its tissue of origin" (Quadro et al. 2004, D'Ambrosio et al. 2011). Supporting this statement, it has been suggested that RBP4 and TTR made in the visceral yolk sac may be involved in transport of retinol and thyroid hormone from the maternal circulation to the developing fetus (Richardson 2009). As mentioned, choroid plexus-derived TTR is thought to mediate the distribution of thyroid hormone to the central nervous system, and furthermore, also may help protect against ischemia-induced brain injury (Robbins et al. 2000, Santos et al. 2010). In the case of islet-derived TTR, it has been theorized that the protein could affect the processing of glucagon, and furthermore that it may influence deposition of islet amyloid, which is found in at least 95% of subjects with Type II diabetes mellitus (Westermark et al. 2008).

An as-of-yet unanswered question raised by the current study is why RBP4 and TTR are expressed within ghrelin cells, especially at such high relative levels. One might postulate that their expression is related to the emerging role of ghrelin cell-derived products, and thus, the ghrelin cell as a whole, in metabolism. As mentioned, acyl-ghrelin, desacyl-ghrelin, obestatin, nesfatin-1 and RBP4 all have been shown (albeit in certain cases, inconsistently) to affect food intake, body weight and/or blood glucose handling. Relationships of RBP4 to some of these

other ghrelin cell products has been examined in at least one human study, in which 6 mo following gastric band surgery, which resulted in a reduction of BMI of on average 6 kg/m^2, circulating levels of total ghrelin increased, obestatin increased and RBP4 decreased(Haider et al. 2007). Whereas low circulating ghrelin has been associated with elevated fasting insulin and insulin resistance in humans (Ikezaki et al. 2002, Poykko et al. 2003), the opposite is true for RBP4 (Graham et al. 2006). Here, regulation of TTR mRNA expression in the stomach by caloric restriction also suggests that ghrelin-cell derived TTR may be involved in metabolism.

Despite the high level of RBP4 mRNA within ghrelin cells, especially as compared to liver and white adipose tissue, its overall contribution to the complement of RBP4 circulating in the bloodstream is seemingly low. The stomach is a relatively small organ, especially as compared to the liver and white adipose tissue, and furthermore, the ghrelin cell population is limited to approximately 0.3–1% cells comprising the entire gastric mucosa (Sakata et al. 2009). Even with the marked expansion of ghrelin cells in the mice harboring ghrelinomas, total circulating RBP4 level remained unchanged. This perhaps suggests that ghrelin cell-derived RBP4 and TTR may not impact whole body insulin resistance and/or thyroid hormone transport. Rather, ghrelin cell-derived RBP4 and TTR instead may have effects that are more localized to the stomach, just as has been suggested for other tissues. Although co-expression of RBP4 with TTR within the ghrelin cell does suggest that there exists a shared function, the roles of ghrelin cell-derived RBP4 and TTR could be unrelated.

In conclusion, relatively high levels of RBP4 and TTR expression by gastric ghrelin cells have been found. Thus, RBP4 and TTR join a short list of other proteins known to be secreted by ghrelin cells, including acyl-ghrelin, desacyl-ghrelin, obestatin and nesfatin-1. At this time, the etiology of their expression within ghrelin cells can only be speculated, although it is assumed that this could be related to an emerging general role for the ghrelin cell in blood glucose handling and metabolism. Alternatively, RBP4 and TTR may have a more regional function

unique to ghrelin cells and their neighboring cells. Future studies in which RBP4 and/or TTR expression can be deleted specifically from ghrelin cells may be helpful in pinpointing the actions of ghrelin cell-derived RBP4 and TTR.

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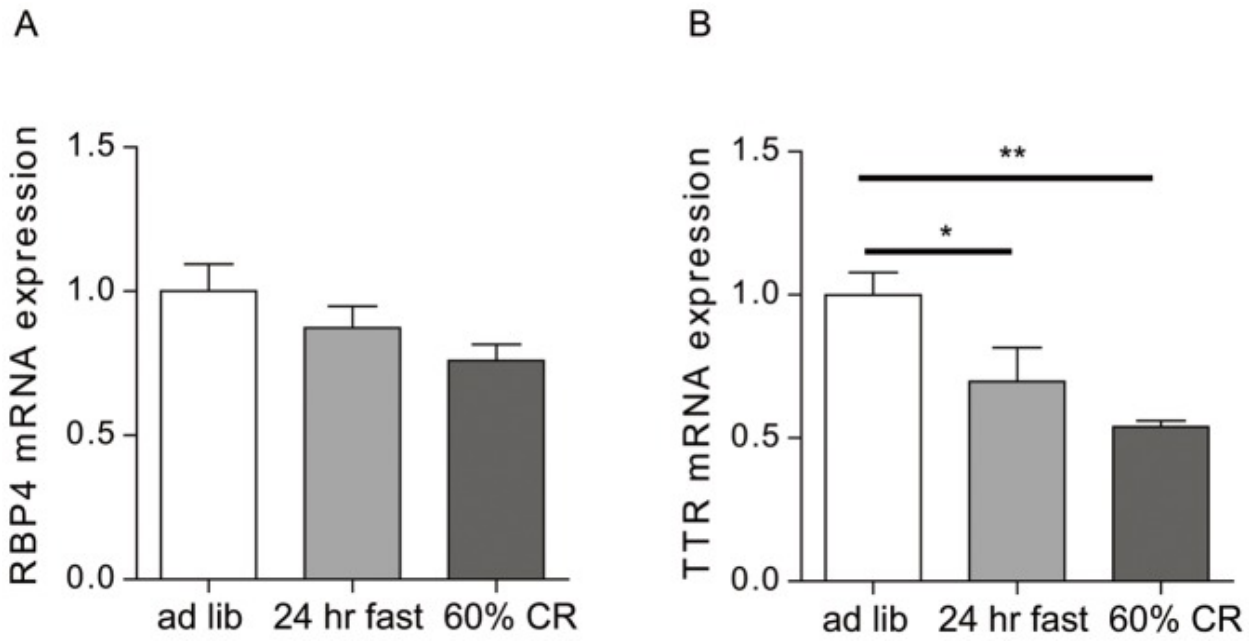

Figure 4.1. Effects of caloric restriction on RBP4 and TTR mRNA levels in the stomach. Relative RBP4 (A) and TTR (B) mRNA levels within the stomachs of ad lib-fed, 24-hr-fasted and chronically food restricted [60% of usual daily calories for 7 days (60% CR)] C57BL/6J mice were determined using qPCR. Statistically significant effects of the two caloric restriction protocols were noted only for TTR (*P<0.05 and **P<0.01).

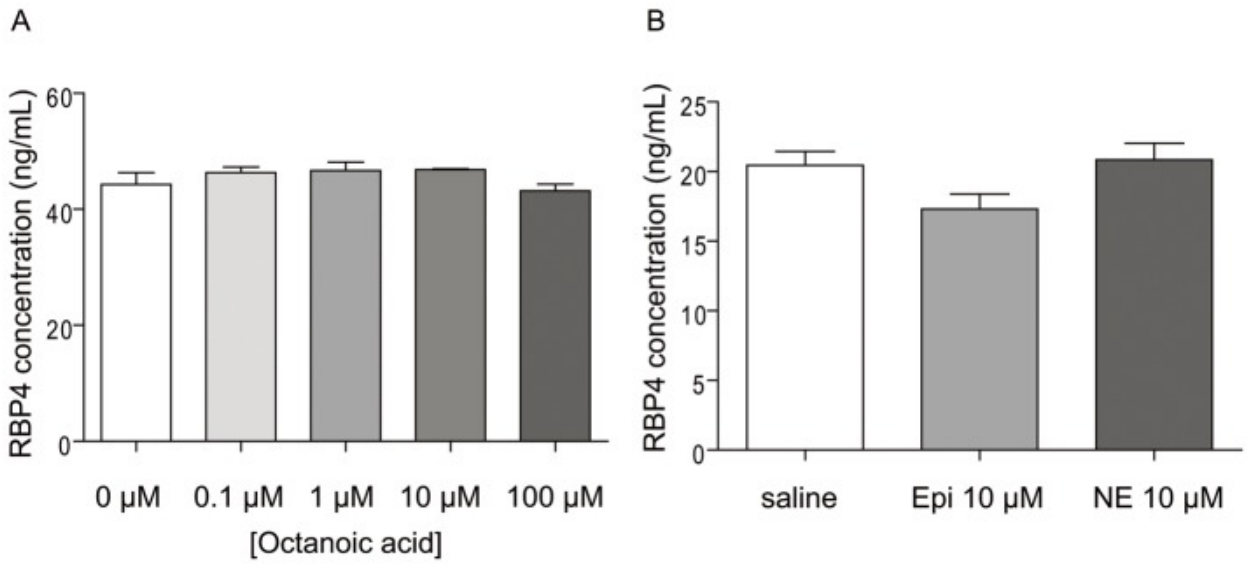

Figure 4.2. The effect of octanoic acid, epinephrine and norepinephrine on RBP4 release from immortalized ghrelin cells. RBP4 protein levels were measured by ELISA in the culture media of PG-1 cells incubated for 6 hrs in the presence of different concentrations of octanoic acid, 10 µM epinephrine (Epi) or 10 µM norepinephrine (NE).

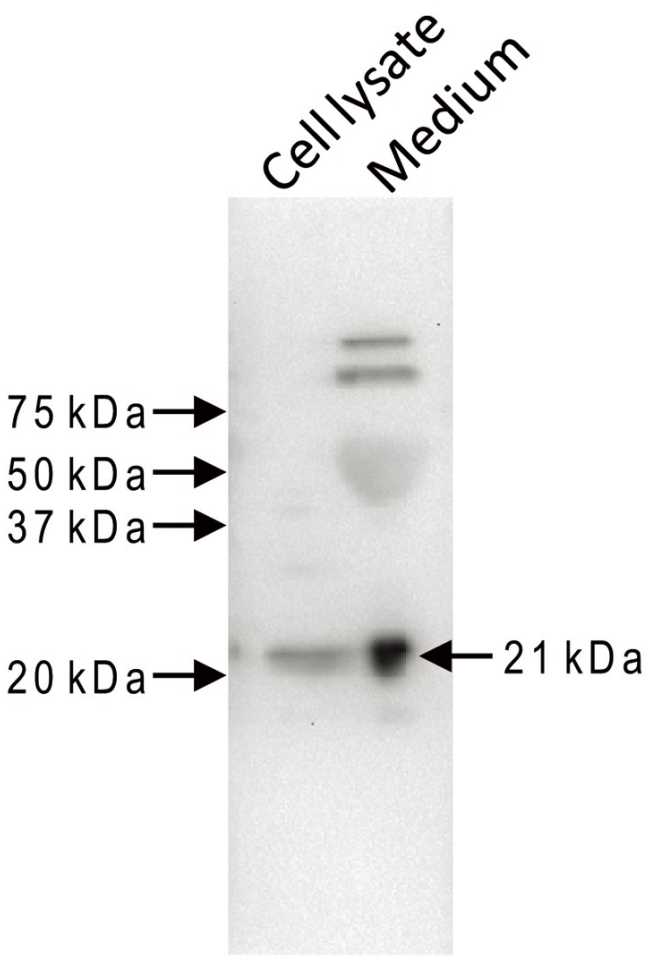

Figure 4.3. Expression of RBP4 by immortalized ghrelin cells. A 21-kDa band corresponding to the expected size of RBP4 protein was detected in SG-1 cell lysate and SG-1 culture media by Western blot analysis using anti human RBP4 antisera. A representative blot is depicted.

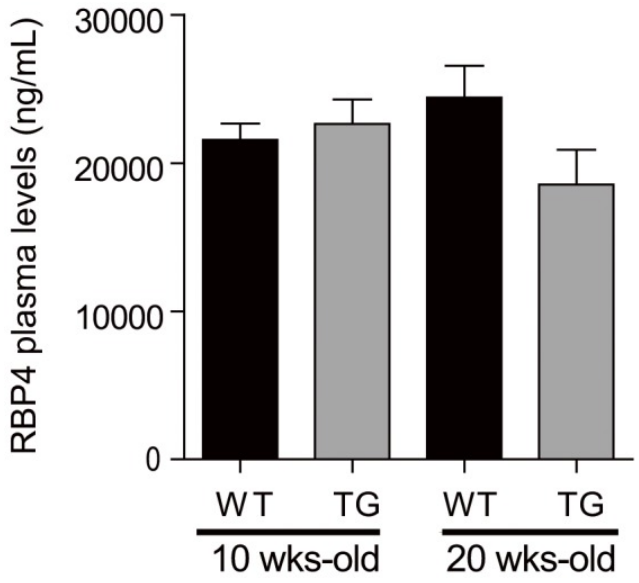

Figure 4.4. Plasma RBP4 concentration in ghrelin-SV40 T-antigen transgenic mice. Plasma concentration of RBP4 in wild type (WT) and ghrelin-SV40 T-antigen transgenic (TG) mice at 10 and 20 wks of age, as measured by ELISA.

mRNA	hrGFP/ghrelin cell-enriched pools signal intensity	hrGFP-negative pools signal intensity	fold-increase
ghrelin	47159	159	137
GOAT	25977	142	169
RBP4	8769	276	42
TTR	8681	543	21
chromogranin A	38699	2679	8
prohormone convertase 1/3	3564	342	11
prohormone convertase 2	3962	55	79
NUCB2	11544	2168	6
RESP18	19795	82	239

*NUCB2 = nucleobindin 2, which encodes nesfatin-1.

Table 4.1 Relative levels of selected set of mRNAs within FACS-separated pools of gastric mucosal cells, as determined by Affymetrix array.

mRNA	whole stomach	hrGFP-negative pools	hrGFP/ghrelin cell-enriched pools	SG-1 ghrelinoma cell line	PG-1 ghrelinoma cell line	liver	white adipose tissue
Ghrelin	1.0	0.03±	1989±	103.0±	56.2±	0.0003±	0.0005±
	(15.95±0.5)†	0.02*	292*	12.6*	13.0*	0.0001*	0.0001*
RBP4	1.0	0.3±	75.9±	249.8±	128.1±	244.6±	80.0±
	(21.8±0.6)	0.2*	11.8*	58.3*	17.6*	14.9*	4.7*
TTR	1.0	0.9±	151.2±	281.2±	473.3±	242.9±	0.004±
	(22.0±0.3)	0.3	71.7*	61.3*	52.9*	62.0*	0.001*
NUCB2	1.0	0.09±	2.9±	3.1±	1.5±	0.07±	6.4±
	(22.4±0.3)	0.04*	0.8	0.5*	0.1	0.001*	0.6*

Table 4.2. Relative levels of mRNAs in various mouse tissues and mouse ghrelinoma cell lines.

Each value represents the amount of mRNA relative to that in whole stomach, which is arbitrarily defined at 1.0 and is shown as the means ± SEM of 3 different preparations. Each determination was done in duplicate.

†Values in parentheses denote the mean ± SEM of threshold cycle values.

*Level significantly different from whole stomach ($P<0.05$).

CHAPTER FIVE

Characterization of gastric and neuronal histaminergic populations using a transgenic mouse model.

Adapted from: **Walker AK**, Park WM, Chuang JC, Perello M, Sakata I, Osborne-Lawrence S, Zigman JM. Characterization of gastric and neuronal histaminergic populations using a transgenic mouse model. (2013). PLoS One. 8(3):e60276.

Abstract

Histamine is a potent biogenic amine that mediates numerous physiological processes throughout the body, including digestion, sleep, and immunity. It is synthesized by gastric enterochromaffin-like cells, a specific set of hypothalamic neurons, as well as a subset of white blood cells, including mast cells. Much remains to be learned about these varied histamine-producing cell populations. Here, I have reported the validation of a transgenic mouse line in which Cre recombinase expression has been targeted to cells expressing histidine decarboxylase (HDC), which catalyzes the rate-limiting step in the synthesis of histamine. This was achieved by crossing the HDC-Cre mouse line with Rosa26-tdTomato reporter mice, thus resulting in the expression of the fluorescent Tomato (Tmt) signal in cells containing Cre recombinase activity. As expected, the Tmt signal co-localized with HDC-immunoreactivity within the gastric mucosa and gastric submucosa and also within the tuberomamillary nucleus of the brain. HDC expression within Tmt-positive gastric cells was further confirmed by quantitative PCR analysis of mRNA isolated from highly purified populations of Tmt-positive cells obtained by fluorescent activated cell sorting (FACS). HDC expression within these FACS-separated cells was found to coincide with other markers of both ECL cells and mast cells. Gastrin expression was co-localized with HDC expression in a subset of histaminergic gastric

mucosal cells. Likely, these transgenic mice will facilitate future studies aimed at investigating the function of histamine-producing cells.

Introduction

Whether assisting with the digestive processes of the stomach, signaling as a neurotransmitter in the central nervous system, or inducing an inflammatory reaction, the biogenic amine histamine plays a major role in numerous behavioral and physiological processes(Rangachari, 1992; Haas et al., 2008). In order to generate histamine, cells utilize the enzymatic activity of histidine decarboxylase (HDC), which acts to decarboxylate the amino acid L-histidine to form histamine (Rosenthaler et al., 1965; Green et al., 1987; Hocker et al., 1996). Since HDC is essential for the proper regulation of histamine formation and release, the ability to identify cells that contain HDC is central to investigating the physiology of the histaminergic systems of the body.

The gastric mucosa of the stomach contains a highly regulated population of histaminergic cells known as enterochromaffin-like (ECL) cells (Lindstrom et al., 2001). Classically, ECL cells have been distinguishable from other gastric mucosal cell populations by the distinctive electron microscopic appearance of their secretory granules (Date et al., 1999; Kamoshida et al., 1999). In rodents, ECL cells store approximately 80% of the total gastric histamine content, and thus, account for the majority of histaminergic cells in the oxyntic mucosa(Andersson et al., 1992). One of the best characterized actions of ECL cell-derived histamine is the stimulation of gastric acid secretion from nearby parietal cells (Hakanson et al., 1991; Lindstrom et al., 2001), which not only plays a pivotal physiologic role in digestion, but also plays a key pathologic role in the occurrence of dyspepsia, gastroesophageal reflux disease, and peptic ulcer disease (Troidl et al., 1976; Barth et al., 1977; Matter et al., 1990;

DeVault et al., 1994; Rackoff et al., 2005). Blockade of H_2-type histamine receptors has long been utilized as one of the major means of treating those ailments and emphasizes the importance of ECL cell-derived histamine (Grossman et al., 1974; Rackoff et al., 2005). Among the important mechanisms found to regulate ECL function, endocrine, paracrine, and neural pathways all help direct ECL cell histamine release (Zhao et al., 2012). Of importance, gastrin stimulation of calcium signaling largely mediates histamine release, and pituitary adenylate cyclase activating peptide (PACAP) regulates ECL cell function through activation of PACAP type 1 receptors (PAC1) (Zeng et al., 1998; Zeng et al., 1999; Zhao et al., 2012). Despite a vast literature on ECL cells, the sparse and dispersed nature of their distribution within the stomach as well as the lack of an identifier more practical than the appearance of their secretory granules under electron microscopy has made study of this cell type challenging.

Mast cells comprise another important population of histamine-producing cells within the stomach(Kurbel et al., 1995). Derived from the bone marrow, mast cells are found not only in the stomach but also in the many connective tissues throughout the body, as well as the brain and dura (Metcalfe et al., 1997; Haas et al., 2008). In the mast cell immune response, immunoglobulin E (IgE) attached to mast cells binds to antigens, thus resulting in degranulation and release of histamine from the cells (Kurbel et al., 1995). In the gastric mucosa, the release of mast cell histamine typically activates vascular H_1 receptors to encourage extravasation and chemotaxis (Kurbel et al., 1995). Mast cells in the stomach have been implicated in contributing to increased gastric acid secretion, irritable bowel syndrome, polyp formation, and tumor angiogenesis (Hodges et al., 2012). Some but not all studies suggest that gastric mast cells also contribute to the development of peptic ulcers, gastric carcinomas, and gastric injury (Rioux et al., 1994; Caruso et al., 2007; Levin et al., 2011). In response to gastrointestinal parasites, mast cells play an important role in host defense, assisting in parasite expulsion and rejection (Gurish

et al., 2004; Meeusen et al., 2005). However, the importance of gastric mast cells in various physiologic as well as pathogenic stomach inflammatory responses has only begun to be explored.

Histamine also serves as an important signal within the central nervous system. Histaminergic neurons are concentrated in a small region of the posterior basal hypothalamus known as the tuberomamillary nucleus (Takeda et al., 1984; Ericson et al., 1987; Inagaki et al., 1990; Haas et al., 2008). As would be predicted from the widespread histaminergic efferent projections from the tuberomamillary nucleus, histamine receptors are found widely distributed throughout the brain (Ericson et al., 1987; Inagaki et al., 1990; Haas et al., 2008). Such suggests a heavy influence of histamine signaling on many different brain regions and on a variety of behaviors (Watanabe et al., 1983; Garbarg et al., 1985). Perhaps the best characterized function of central histamine is related to its effects on arousal state: activation of histaminergic neurons increases wakefulness while inhibition of these neurons has a sedating effect (Haas et al., 2008). CNS histamine also regulates several other homeostatic processes, including thermoregulation, feeding, drinking, energy metabolism, nociception, and biological rhythms, to name a few, and also influences higher brain functions such as mood and cognition (Haas et al., 2008). With such an extensive range of physiological and behavioral effects, the idea of histamine neurons in the tuberomamillary nucleus as distinct subpopulations rather than a single unit of cells is beginning to gain interest (Blandina et al., 2012). Of note, the histaminergic neuron system has historically been divided into five distinct cell clusters based on regional location within the tuberomamillary nucleus, termed E1–E5, and several recent studies suggest the existence of functional distinctions between these five nuclei (Ericson et al., 1987; Wada et al., 1991; Karlstedt et al., 2001; Blandina et al., 2012). To elaborate, different types of stress such as restraint stress, foot shock, or hypoglycemia have been found to activate

histamine neurons in the rostral subgroups (E4–E5) rather than the caudal subgroups (E1–E3), while CO_2 exposure or acidification in rat brain slices causes excitation in the ventrolateral E2 neurons (Miklos et al., 2003; Johnson et al., 2005; Yanovsky et al., 2012).

Although several key functions and regulatory pathways already have been established regarding histaminergic systems of the brain and stomach, much remains to be discovered. Recently, the lab reported the generation of a novel HDC-Cre mouse model in which Cre recombinase is expressed within histidine decarboxylase-containing cells (Yanovsky et al., 2012). Previously, co-expression of Cre recombinase activity within about 74% of ventrolateral (E2) HDC-immunoreactive neurons HDC was reported (Yanovsky et al., 2012). However, expression of the transgene was not explored in the other four tuberomamillary nucleus subgroups or in the stomach for that initial study. The aim of the current study was to more fully validate and quantify the specific expression of Cre recombinase in the stomach and brains of our HDC-Cre mouse model. Indeed, Cre recombinase activity localized within the vast majority of HDC-immunoreactive cells populating the stomach and the various tuberomamillary nucleus subgroups. Furthermore, HDC expression within highly purified populations of gastric mucosal cells expressing the transgene, as made possible by fluorescence activated cell sorting, was found to coincide with other markers of both ECL cells and mast cells. The collective findings suggest that these transgenic mice will facilitate future studies aimed at investigating the function of histamine-producing cells.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the guidelines included in the Guide and Care for Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center under the permit numbers 2009-0377 and 2008-0107. Methods to minimize suffering were performed such as the use of chloral hydrate for anesthetization of animals during non-survival surgeries.

Generation and Care of HDC Reporter Mice

HDC-Cre transgenic mice were generated by microinjection of a Cre-modified, mouse HDC bacterial artificial chromosome into pronuclei of fertilized one-cell stage embryos of C57BL6/J mice. The construction of the HDC-Cre transgene is described in more detail elsewhere (Yanovsky et al., 2012), but in brief involved replacing a portion of the HDC gene encoding the first 47 amino acids of HDC and the interventing intron 1 with the coding sequence of Cre recombinase. Such was accomplished in an HDC bacterial artificial chromosome (RP24-141N14; BACPAC Resources Center at Children's Hospital Oakland Research Institute) which spanned the entire coding region of HDC plus approximately 92.42 kb sequence upstream of the HDC start codon and approximately 13.91 kb sequence downstream of the HDC stop codon (Yanovsky et al., 2012). As mentioned, an initial characterization of this novel transgenic line, limited to confirmation of Cre recombinase activity in the ventrolateral tuberomamillary nucleus, was previously described (Yanovsky et al., 2012). For all of the studies performed in the current report, HDC-Cre transgenic mice were bred to Rosa26-lox-STOP-lox-tdTomato reporter mice [B6J/N.Cg-Gt(ROSA)26Sortm14(CAG–tdTomato); stock #007908; The Jackson Laboratory, Bar Harbor, Maine], in which a transcriptional stop cassette is removed only in the presence of Cre recombinase activity, thus resulting in the expression of tdTomato (Tmt) fluorescence. Mice with

one copy of each transgene are herein designated as HDC/Tmt mice. Animals were fed standard chow diet (Teklad Global Diet 16% Protein Diet [2016]; Harlan Teklad, Madison, WI). They had free access to water and were housed under 12 hours of light/12 hours of dark per day in a temperature-controlled environment.

Tissue Preparation for Immunohistochemistry

Tissue preparation for histological experiments was performed as described previously (Sakata et al., 2009). Briefly, for immunohistochemistry, adult (8–15 weeks old) mice were anesthetized with chloral hydrate (500 mg/kg), injected intraperitoneally, and then perfused transcardially with diethylpyrocarbonate (DEPC)-treated 0.9% saline followed by 10% neutral buffered formalin. The stomachs and brains were removed, and after being stored in formalin for 4–6 hours at 4°C, were placed into 30% sucrose-DEPC-treated phosphate buffered saline (PBS) overnight. Stomachs were then embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd, Tokyo) and sectioned transversely at a thickness of 12 µm onto SuperFrost slides (Fisher Scientific, Pittsburg, PA) using a cryostat. Five equal series of 20 µm-thick coronal brain sections were collected using a sliding microtome and stored at −20°C in cryoprotectant until use for immunohistochemistry.

Histochemistry of Stomach Sections

Visualization of Tmt Fluorescence

Slides with stomach sections were coverslipped with Vectashield Hardset Mounting Media (Vector Labs, Burlingame, CA) and examined under an Olympus IX51 microscope (Center Valley, PA), and photomicrograph images were taken of endogenous Tmt fluorescence using the Olympus DP Controller program.

Immunohistochemistry for HDC

After air-drying at 4°C overnight, the slides with stomach sections were washed in PBS three times and pretreated with 1% sodium dodecyl sulfate (SDS)-PBS for 5 min at room temperature. The slides were incubated with a rabbit polyclonal anti-HDC antibody (1:500; Cappel, Aurora, OH) overnight at room temperature. After washes with PBS, the slides were incubated in Alexa Fluor 488® donkey anti-rabbit IgG (Molecular Probes, Carlsbad, CA) for 1 hr at room temperature. Labeled samples were coverslipped with Vectashield HardSet Mounting Media (Vector Labs, Burlingame, CA), and examined under a Zeiss fluorescence microscope with ApoTome attachment (Zeiss Axio Imager Z1; Thornwood, NY). Photomicrograph images were taken using AxioVision software. The specificity of the immunohistochemistry reaction was tested by omitting the primary antibody; no labeling was detected in these control reactions.

Toluidine Blue Histochemistry

The Olympus IX51 microscope was used to visualize endogenous Tmt fluorescence within slide-mounted stomach tissue sections for prior to staining. Mast cells were identified based on their Tmt fluorescence and their characteristic oval-shaped, “fried egg” appearance, in which a dim fluorescent cytoplasm appears surrounding a more intensely fluorescent nucleus (Mysore, 2010). Photomicrograph images of the mast cells were taken and the locations of the cells were carefully noted in order to pinpoint the same cells after staining. Slides with sections were then incubated in a Toluidine blue solution comprised of Toluidine blue O (Sigma-Aldrich, St. Louis, MO) in 70% ethanol and 1% NaCl (pH 2.3). After 3 min, sections were washed in distilled water, and then dehydrated with 95% and 100% ethanol, followed by xylene and coverslipping. After staining, cells which were captured in the photomicrograph images taken before the toluidine blue staining protocol were again visualized under brightfield on the microscope. New photomicrograph images were taken to capture Toluidine blue staining in those cells that had previously displayed Tmt fluorescence.

Dual-label Immunohistochemistry for HDC and Gastrin

For double labeling, slides were pretreated with 1% sodium dodecyl sulfate (SDS)-PBS for 5 min at room temperature prior to being incubated simultaneously with a guinea pig polyclonal anti-gastrin antibody (1:500, Acris, San Diego, CA) and the rabbit polyclonal anti-HDC antibody (1:500). After washes with PBS, the slides were incubated in Alexa Fluor 488® donkey anti-rabbit IgG (Molecular Probes, Carlsbad, CA) together with Alexa Fluor 594® goat anti-guinea pig IgG (Molecular Probes) for 1 hr at room temperature. After coverslipping with Vectashield HardSet Mounting Media (Vector Labs, Burlingame, CA), slides were examined under a Zeiss fluorescence microscope with ApoTome attachment (Zeiss Axio Imager Z1; Thornwood, NY), and photomicrograph images were taken with the Axiovision software. The specificity of the immunohistochemistry reaction was tested by omitting the primary antibody; no labeling was detected in these control reactions.

Stomach Mucosal Cell Isolation and Fluorescence Activated Cell Sorting (FACS)

A combined enzymatic and mechanical dispersion method was used to prepare isolated stomach mucosal cells, as previously reported (Sakata et al., 2006; Sakata et al., 2009). Briefly, mice were quickly killed with cervical dislocation and the stomachs were removed, inverted and inflated. The inflated stomachs were incubated in 2.4 U/mL Dispase II solution (Roche Diagnostic Corporation, Indianapolis, IN) for 1.5 hours. A secondary enzymatic digestion of cell suspensions with 0.25% trypsin-EDTA (Gibco, Gaithersburg, MD) for 5 min was followed by filtration through a 100 µm nylon mesh. Cells were re-suspended in FACS buffer (3% FBS, 0.5 mM EDTA, 0.1% BSA, 10 U/mL DNaseI, 20 mg/mL glucose in HBSS) and re-filtered through a 35 µm filter right before the sort. Dissociated single cells obtained from HDC/Tmt mice were separated by a FACS Aria (Becton Dickinson, San Jose, CA) at the UTSW Medical Center Flow Cytometry Multi-user Core Facility. Cells were sorted into a Tmt-enriched population and a Tmt-

negative population based on size, complexity and intensity of Tmt fluorescence (at 585 nm) and fluorescence at 530 nm. Ten independent FACS preparations (4–7 mice consisting of both males and females were used for each preparation) were included in the subsequent analyses. Depending on the preparation, these Tmt-enriched and Tmt-negative pools corresponded to slightly different percentages of sorted, living cells. The Tmt-enriched pools corresponded to 0.3% to 1.1% of sorted living cells, and the Tmt-negative pools corresponded to 20% to 97% of sorted, living cells. For “mucosa” samples, non-sorted, dissociated cells from C57BL/6J mice were obtained using the same protocol minus the FACS separation.

RNA Extraction and Quantitative PCR

The method for RNA extraction of FACS-sorted cells and quantitative PCR was similar to that reported previously (Sakata et al., 2009; Chuang et al., 2011). The Tmt-enriched and Tmt-negative pools were adjusted after each FACS separation to contain a matched number of cells ranging from 20,000 to 50,000, and then were submitted to the RNA extraction protocol below. For the whole mucosa preparations, the entire populations of non-sorted gastric mucosal cells from each of 3 separate C57BL/6J mice were independently submitted to the RNA extraction method below. Total RNA was extracted using centrifugation with STAT60 for lysing of the cells, phenol-chloroform for phase separation, and isopropanol for precipitation of the pellet. The RNA was incubated in isopropanol at 4°C overnight prior to centrifugation in order to maximize the amount of total RNA precipitated. Lastly, RNA pellets were washed with 75% ethanol in DEPC-PBS, reconstituted in RNAlater (Ambion, Naugatuck, CT), and the concentration and relative purity of the RNA was determined using a NANODROP 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA was stored at −80°C until use. After DNase I (Quiagen, Valenica, CA) treatment, complementary DNA (cDNA) was synthesized from 500 ng–1 µg total RNA using Superscript III reverse transcriptase (Invitrogen).

For qPCR, 25 ng cDNA was loaded per well with iTaq SYBR Supermix (Bio-Rad Laboratories, Hercules, CA) for amplification and detection; mRNA levels for genes of interest were measured with an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster, City, CA). Previously designed and validated primers are as follows (Sakata et al., 2009): HDC : *5′-GCGACCCTTCCTTCGAAATT-3′*; *5′-CCTTTAACACACTTTCTGTGAGACAAT-3′*, tryptophan hydroxylase 1: *5′-CCTTGGAGCTTCAGAGGAGA-3′*; *5′- CAGCTGTCCATCTTGTTTGC-3′*, gastrin: *5′-TCCAGGGTCCTCAACACTTC-3′*; *5′-CCAAAGTCCATCCATCCGTA-3′*, ghrelin: *5′-GTCCTCACCACCAAGACCAT-3′*; *5′-TGGCTTCTTGGATTCCTTTC-3′*, chromogranin A: *5′-GCAGGCTACAAAGCGATCCA-3′*; *5′-CTCTGTCTTTCCATCTCCATCCA-3′*, prohormone convertase1/3: *5′-GGCACCTGGACATTGAAAATTAC-3′*; *5′-TTCATGTGCTCTGGTTGAGAAGA-3′*, prohormone convertase 2: *5′-CAAGCGGAACCAGCTTCA-3′*; *5′-ATTCCAGGCCAACCCCA-3′*, H+/K+ ATPase β-subunit: *5′-CCCAGCTTCGGCTTCGA-3′*; *5′-TGGAGACTGAAGGTGCCATTG-3′*, gastric intrinsic factor: *5′-GAAAAGTGGATCTGTGCTACTTGCT-3′*; *5′-AGACAATAAGGCCCCAGGATG-3′*, pepsinogen F: *5′-GAAGTGGCTCTGGGTCCTT-3′*; *5′-GGCTTTCCCGCAGGTTTT-3′*, calpain 8: *5′-TTCTGCCTGAGGGTGTTCTC-3′*; *5′- TCTTCTCCATCCATGTCACG-3′*, *′*; *5′-CCTTTAACACACTTTCTGTGAGACAAT-3′*, somatostatin: *5′-CCCAGACTCCGTCAGTTTCT-3′*; *5′-GGGCATCATTCTCTGTCTGG-3′*, and cyclophilin: *5′-TGGAGAGCACCAAGACAGACA -3′*; *5′-TGCCGGAGTCGACAATGAT-3′*.

Newly designed primers were created to span exon-exon junctions to reduce amplification of contaminating DNA, and the efficiency and specificity of these primer pairs were confirmed by generating titration slopes and dissociation curves. These primers are as follows: Tpsb2: *5′-CTTCCCCCAGGGACATC-3′*; *5′-GGAGAGGCTCGTCATTATCAATG-3′*, PAC1: *5′-GCAGTCATTGCTTCGTTTCCA-3′*; *5′-AGAGGTATAGGCCTTCAATGAACAG-3′*, and Tmt *5′-CACCATCGTGGAACAGTACGA-3′*; *5′-GCCATGCCCCAGGAACA-3′*.

The PCR protocol included an initial template denaturation for 3 min at 95°C. The cycle profiles were programmed as follows: 15 sec at 95°C (denaturation) and 45 sec at 60°C (annealing and extension). Forty cycles of the profile were run and cyclophilin was used for normalization.

Of note, a separate quantitative PCR analysis was performed to confirm the appropriateness of using cyclophilin as the housekeeping gene for normalization of results. Two independent FACS preparations (5–6 mice consisting of both males and females were used for each preparation) were included. As above, equal amounts of cDNA (25 ng) prepared from each of the two new sets of Tmt-enriched and Tmt-negative pools were amplified with primers for cyclophilin and two other housekeeping genes, β-actin (*5′- CATCGTGGGCCGCCCTA -3′*; *5′- CACCCACATAGGAGTCCTTCTG -3′*) and GAPDH (*5′- CAAGGTCATCCATGACAACTTTG -3′*; *5′- GGCCATCCACAGTCTTCTGG -3′*). The mean threshold cycle values for cyclophilin in the Tmt-enriched and Tmt-negative pools were essentially the same, without a statistically significant difference (19.80±0.26 and 19.66±0.29, respectively). Similar threshold cycles for β-actin and GAPDH were also observed in the Tmt-enriched and Tmt-negative pools (16.12±0.07 and 16.28±0.03, β-actin; 17.51±0.23 and 17.04±0.07, GAPDH).

Immunohistochemistry of Brain Sections

Brain tissue sections were mounted onto SuperFrost slides with PBS and allowed to dry overnight prior to staining. Slides with tissue sections were rehydrated in PBS three times, pretreated with 1% SDS-PBS for 12 min, and washed three times in PBS (total 45 min). Slides were then incubated in 3% normal donkey serum (Equitech-Bio, Kerrville, TX) with 0.3% Triton X-100 in PBS for 1 hr, followed by an overnight incubation at room temperature in rabbit anti-HDC antiserum (1:500, Cappel) in 0.3% Triton X-100-PBS at a concentration of 1:50. After washing in PBS, slides were incubated in Alexa Fluor 488® donkey anti-rabbit IgG (1:300;

Molecular Probes, Carlsbad, CA) for 1 hr at room temperature. Following some final PBS washes, sections were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and viewed using microscopy. Tmt fluorescence was visualized using the Zeiss Axioskop 2 microscope and was quantified in sections after the stain was performed in order to determine co-localization. Photomicrograph images were taken using Axiovision software. Omission of the primary antibody as a control experiment revealed a lack of staining in these tissues.

Analysis of Data

The method for quantification of co-localization was carried out as performed previously (Sakata et al., 2009). To determine the degree of overlap between Tmt-positive cells and HDC immunoreactivity in the stomach, four different section planes of oxyntic mucosa, each separated by ≥70 μm along the extent of the gastric corpus, were viewed in each of five mice. To determine the degree of overlap between HDC-immunoreactive cells and gastrin-immunoreactive cells, eight different section planes of the gastric antrum were viewed in each of three mice. At each section plane, analyses were done with both 20X and 40X Zeiss Plan-Apochromat objectives from at least five fields, each 480 μm×480 μm in area, over the whole circumference of the stomach. For visualization of brain tissue sections, every fifth brain section containing the tuberomamillary nucleus was analyzed in three mice, and co-localization of HDC immunoreactivity and Tmt fluorescence was determined in the histaminergic regions E1–E5. Analysis of both the stomach and brain consisted of counting the total number of Tmt-fluorescent cells, HDC-immunoreactive cells, and cells with co-localization of HDC-immunoreactivity and Tmt fluorescence. Quantification was performed manually while visualizing live through the microscope. Using these cell counts, the percentage of HDC-immunoreactive cells containing Tmt fluorescence was calculated ([# of cells with co-localization/total # of HDC-immunoreactive cells] x 100); the percentage of Tmt-fluorescent cells

with HDC-immunoreactivity was also calculated ([# of cells with co-localization/total # of Tmt-fluorescent cells] x 100). Images of sections were taken using either the green filter, the red filter, or both. Adobe PhotoShop 7.0 (San Jose, CA) was used to adjust contrast, brightness, and color of the photomicrographs.

For qPCR analysis, one-way ANOVAs were performed; Dunnett's post-hoc analysis was used to compare expression levels in the sorted cell populations with those in whole mucosa. Statistics were performed using GraphPad Prism 5.0. $P<0.05$ was considered statistically significant, and all data are expressed as mean ± standard error of the mean.

Results

Stomach expression of Cre recombinase activity

Transgenic HDC-Cre mice were bred with Rosa26-lox-STOP-lox-tdTomato reporter mice in order to generate offspring carrying the two transgenes (HDC/Tmt mice). The removal of the lox-flanked transcriptional STOP cassette by Cre recombinase results in the expression of the fluorescent Tmt protein specifically in cells expressing Cre recombinase. As Cre recombinase expression is directed by HDC transcriptional regulatory elements, Tmt should report on the location of histamine-producing cells. Fluorescence microscopy was utilized to verify the expected specificity of Cre recombinase activity within histaminergic cells of HDC/Tmt mouse stomachs. Three distinct cell types with Tmt fluorescence were observed in the stomach (Figure 5.1). Most labeled cells within the gastric mucosa were intensely fluorescent and displayed a mostly elongated (not-rounded) morphology, which is consistent with the known shape of ECL cells (Figure 5.1, leftward-facing arrow) (Chen et al., 1998; Kamoshida et al., 1999). These intensely fluorescent, elongated cells were localized mainly to the glandular bases, with a fewer number of additional cells scattered within the glandular necks and apices. Such was consistent with previously published descriptions of enterochromaffin-like (ECL) cell distribution

(Kamoshida et al., 1999). Within the gastric submucosa, muscularis, and less often, the mucosa, a generally weaker fluorescence was also observed within an oval-shaped cell type that had the appearance of a “fried egg”, with a dim fluorescent cytoplasm surrounding a more intensely fluorescent nucleus, as is commonly observed for mast cells (Figure 5.1, rightward facing arrowhead) (Mysore, 2010). The Tmt fluorescence was much brighter for the elongated, ECL cells (resulting in a yellowish, over-exposed center) than for the oval-shaped mast cells. In the submucosa, muscularis, and occasionally also the mucosa, there was a third population of weakly fluorescent cells with a tiny round shape, smaller than the mast cells (Figure 5.1, upward-facing arrow).

In order to confirm an HDC-specific expression of Cre recombinase activity, co-localization of HDC-immunoreactivity with Tmt fluorescence (as a reporter for Cre recombinase activity) was performed on stomach sections from HDC/Tmt mice (Figure 5.2; Table 5.1). Co-localization of HDC-immunoreactivity with Tmt fluorescence in gastric mucosal cells was quantified, revealing that nearly all (94.5% of) cells with Tmt fluorescence were also immunoreactive for HDC, while 73.4% of cells immunoreactive for HDC displayed Tmt fluorescence (Table 5.1). All three Tmt-fluorescent cell types were included in the co-localization analysis. Of note, Figure 5.2, depicts co-localization within the elongated ECL cells alone; for these photomicrographs, the optics were chosen to clearly distinguish the outlines of the ECL cells, and as such, the intensity of the fluorescence was lowered (as compared to that used in Figure 5.1), such that the more weakly-fluorescent, oval/fried egg-shaped mast cells and the tiny, weakly-fluorescent round cells were no longer visualized. None of the tiny round cells contained HDC-immunoreactivity, and as yet, the identity of these cells is unclear.

With the aim of further verifying that mast cells in the stomach display Tmt fluorescence, toluidine blue staining was performed on stomach sections of HDC/Tmt mice (Figure 5.3). Toluidine blue binds to heparin in the cytoplasmic granules of mast cells, thus distinctly labeling them a blue or purple color. Toluidine blue staining was observed in nearly all the Tmt fluorescent cells with the oval/fried egg-shaped appearance (Figure 5.3). Toluidine blue did not stain the more intensely fluorescent, elongated ECL cells of the mucosa, nor did it stain the weakly-fluorescent tiny round cells within the mucosa, submucosa and muscularis.

Quantitative PCR on FACS-separated gastric mucosal histaminergic cells

To further characterize the gastric mucosal histaminergic cells, the Tmt fluorescence signal present in the HDC/Tmt mice was again taken advantage of, this time as a means of isolating an enriched population of these cells. Cells comprising the gastric mucosa of HDC/Tmt mice were first enzymatically and mechanically dispersed and subsequently were submitted for FACS analysis to generate a population of enriched histaminergic cells. This was repeated for a total of 10 separate preparations of gastric mucosal cells. Cells displaying the highest intensity fluorescence were collected as “Tmt-enriched” pools, while cells with the lowest intensity fluorescence were collected as “Tmt-negative” pools (Figure 5.4). Next, mRNA was isolated from these FACS-separated populations, and the expression levels of various gastric endocrine cell, gastric non-endocrine cell, and mast cell markers were determined using quantitative RT-PCR; mRNAs of interest also were determined in samples of non-sorted, whole mucosa preparations (Table 5.2).

As expected, Tmt mRNA was found to be highly expressed within the Tmt-enriched pools as compared to that within Tmt-negative pools, suggesting a successful FACS enrichment of targeted cells. High levels of HDC expression were observed in the Tmt-enriched

populations, verifying that the cells marked with Tmt indeed express significant levels of HDC. Significant elevations in PACAP type 1 receptor (PAC1) a marker for ECL cells, and tryptase beta 2 (Tpsb2), a marker for mast cells, were observed in Tmt-enriched populations (Oh et al., 2005; Lambrecht et al., 2006; Sommerhoff et al., 2007). Common markers of endocrine lineage such as chromogranin A, prohormone convertase 1/3, and prohormone convertase 2 also were present at elevated levels in Tmt-enriched populations. As expected, there was no significant enrichment in the expression of markers for common non-endocrine gastric mucosal cell types such as calpain-8 (a marker of mucus-secreting pit cells), H^+/K^+ ATPase β-subunit (a marker of parietal cells), or gastric intrinsic factor and pepsinogen F (markers of zymogenic and parietal cells) (Shao et al., 1998; Mills et al., 2003; Hata et al., 2006). There also was no significant enrichment in the expression of markers for two separate gastric endocrine cells types, including ghrelin (a marker for X/A-like, ghrelin cells) and somatostatin (a marker for D cells) (Schubert, 2004; Stengel et al., 2010).

Interestingly, within the Tmt-enriched pools, a high amount of tryptophan hydroxylase 1 mRNA was detected. Tryptophan hydroxylase 1 catalyzes the rate-limiting step in the synthesis of serotonin, and thus is commonly used to distinguish serotonergic EC cells from other gastric mucosal endocrine cell types. That said, tryptophan hydroxylase 1 also is found in mast cells, which are an expected component of the Tmt-enriched pools and which produce not only histamine, but also serotonin (Mathiau et al., 1994; Kojima et al., 2000).

Also noted within Tmt-enriched pools was elevated expression of gastrin mRNA, which is a marker for yet another gastric mucosal endocrine cell type, the G-cell. Gastrin and HDC co-localization has been previously described in the rat stomach (Hunyady et al., 1998), although to our knowledge, not in mice. In order to verify that HDC and gastrin co-localization is a usual occurrence in mice that are not genetically manipulated, dual-label immunohistochemistry on

stomachs from C57Bl6/J mice was performed for HDC and gastrin. Indeed, within the gastric antrum (the distal part of the stomach), a small number of HDC-immunoreactive cells also demonstrated gastrin-immunoreactivity, and vice versa, supporting the existence of a subset of histaminergic gastric cells that co-express gastrin (Figure 5.5; Table 5.3)

Characterization of HDC-expressing cells in the histaminergic regions of the brain

Along with the gastric mucosa of the stomach, co-localization of HDC-immunoreactivity and the reporter of Cre recombinase activity (Tmt fluorescence) in HDC/Tmt mice was quantified in the brain (Figure 5.6 and Table 5.4). Percentages of co-localization were determined for each of the five distinct histaminergic regions within the tuberomamillary nucleus of the brain: regions E1–E5 (Karlstedt et al., 2001). Of note, previously the lab reported the expression of Cre recombinase activity in the brain of HDC/Tmt mice, although this determination was restricted to the ventrolateral tuberomamillary nucleus (E2 region) alone. The caudal E3 group represented the tuberomamillary nucleus region with the highest percentage of HDC-immunoreactive cells also exhibiting Tmt fluorescence (82.1%; Table 5.4). Within the lateral histaminergic regions, E1 and E2, 80.8% and 81.8% of HDC-immunoreactive cells also exhibited Tmt fluorescence, respectively. The more medial histaminergic regions E4 and E5 displayed lower levels of co-localization, with 54.9% and 43.2% of HDC-immunoreactive cells also showing Tmt-fluorescence, respectively. Nearly all of cells with Tmt fluorescence within regions E1–E5 displayed HDC immunoreactivity, with % co-localization ranging from 90.6% to 97.5%.

Of note, when viewing sections throughout the whole brain, Tmt expression was observed in several cells localized to the dorsal lateral geniculate nucleus (DLG) of the thalamus and the posterior thalamic nuclei, as well as rare hippocampal cells (Figure 5.7).

These regions are not known to, nor did they in the current study, express HDC-immunoreactivity, suggesting likely non-specific Cre recombinase expression in these regions.

Discussion

Histamine serves as an important signaling molecule for a group of neurons localized to the tuberomamillary nucleus of the brain, for ECL endocrine cells populating the gastric mucosa, and for mast cells and the related basophils which form an important part of the immune system. As mentioned, these histaminergic systems play key roles in many different processes, including sleep, digestion and host immune defense. The sparsely distributed expression of these different populations has undoubtedly hampered a more rapid exploration of the normal physiology and pathophysiology of these systems. The HDC-Cre transgenic mouse model described here is capable of being a useful tool for studying these multiple histaminergic regions and systems. In the present study, a combination of histochemistry and fluorescence activated cell sorting has been used together with quantitative PCR to validate the appropriate expression of Cre recombinase activity within the histaminergic cells populating the brain and stomach. By breeding the HDC-Cre mice with td-Tomato reporter mice, it was easy to visualize cells expressing Cre recombinase activity in the form of Tmt fluorescence. Within the stomachs of HDC/Tmt mice, those cells exhibiting the brightest Tmt fluorescence in the mucosal layer were mainly localized to the base of the glandular region and had an elongated morphology reminiscent of the distribution and shape of ECL cells (Chen et al., 1998). The success of directing Cre recombinase expression to the vast majority of gastric histaminergic cells was achieved, as 73.4% of mucosal cells containing HDC-immunoreactivity also exhibited Tmt fluorescence. Taking advantage of the fluorescent labeling of the histaminergic cells in the gastric mucosa, highly enriched populations of histaminergic cells were isolated using FACS, of which subsequent qPCR analyses confirmed the presence of known markers for both ECL cells

and mast cells. Gastric mast cell expression of Cre recombinase activity was confirmed by co-localization of the tdTomato signal with toluidine blue staining, which is a known marker of mast cells. Expression of Cre recombinase activity within all the various regions of the tuberomamillary nucleus of the brain was also demonstrated. Greater than 80% of the histaminergic neurons of the E1, E2 and E3 regions of the tuberomamillary nucleus contained Cre recombinase activity while less of the histaminergic neurons of the E4 and E5 regions (55% and 43%, respectively) co-localized with Cre recombinase activity. Presumed ectopic Cre recombinase expression that was not co-localized with HDC-immunoreactivity was observed within a small region of the thalamus, rare hippocampal neurons and an otherwise un-identified population of tiny, round cells within the gastric submucosa, gastric muscularis layer, and occasionally, the gastric mucosa. It is possible, although unlikely, that these non-specific populations of fluorescent cells result from embryonic HDC activity that is no longer present in adult cells. This is especially true for the tiny, round gastric cells, as HDC expression has been found to be undetectable in the stomach during the embryonic stages of development (Karlstedt et al., 2001). Additionally, although the tiny, round cells are likely to have been separated into the Tmt-enriched pools of mucosal cells, the histochemical analysis depicting only rare occurrences of these cells within the mucosa suggests that they are not contributing substantively to the presented quantitative PCR analyses.

One curious observation revealed during the validation of the HDC-Cre mouse model was the finding of relatively high gastrin expression in the Tmt-enriched population of isolated gastric mucosal cells. Although gastrin and HDC co-localization had previously been reported in the rat stomach, to our knowledge, this finding has not been replicated in the mouse or elaborated upon since the initial discovery (Hunyady et al., 1998). It might be interesting for future studies to determine if these histamine- and gastrin-co-expressing cells have an electron microscopic appearance more similar to ECL cells or more similar to G-cells as well as to

determine if they serve functions distinct from those ECL cells or G cells that express either histamine or gastrin alone.

The utility of the now validated HDC-Cre transgenic mouse model is potentially broad. As mentioned, Cre-mediated expression of Tmt fluorescence has already permitted ventrolateral tuberomamillary nucleus histaminergic cells to be studied electrophysiologically (Yanovsky et al., 2012). Now that expression of Cre recombinase within histaminergic neurons also has been confirmed in the other regions of the tuberomamillary nucleus, electrophysiological properties of those neuronal subpopulations also can be examined. The concept of functionally divergent populations as opposed to one cohesive population of histaminergic neurons creates exciting possibilities for a new understanding of histaminergic signaling in the central nervous system. Combining the selective expression of Cre recombinase activity with electrophysiology as well as histochemistry and other biochemical techniques can also be taken advantage of to help further clarify the neuroanatomical circuits, physiological processes and behaviors in which these various tuberomamillary regional populations participate. Similarly, within the stomach, the capability to easily detect ECL cells and mast cells using this new mouse tool opens up the possibilities for novel, yet practical studies. The validation of an HDC reporter mouse that accurately reflects histaminergic cells in the stomach will allow researchers in the field to feasibly pursue studies of ECL function, morphology, distribution, gene expression in response to an endless number of stimuli or treatments.

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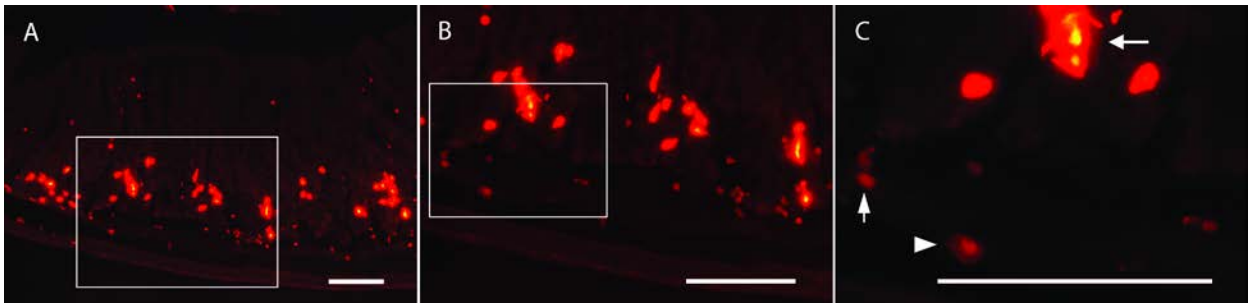

Figure 5.1. Expression of Tmt within the gastric oxyntic mucosa and submucosa of HDC/Tmt mice. A) Low magnification photomicrograph showing expression of cells with different intensities of Tmt fluorescence in the gastric mucosa, submucosa, and muscularis layers. B–C) High magnification of same section reveals distinct shapes of different cell types: leftward-facing arrow points to a representative intensely-fluorescent, elongated cell (ECL cell); rightward-facing arrowhead points to a representative weakly-fluorescent, oval-shaped cell with a “fried egg” appearance (mast cell); upward-facing arrow points to a tiny, weakly-fluorescent round cell. Scale bar is 100 µm in all panels.

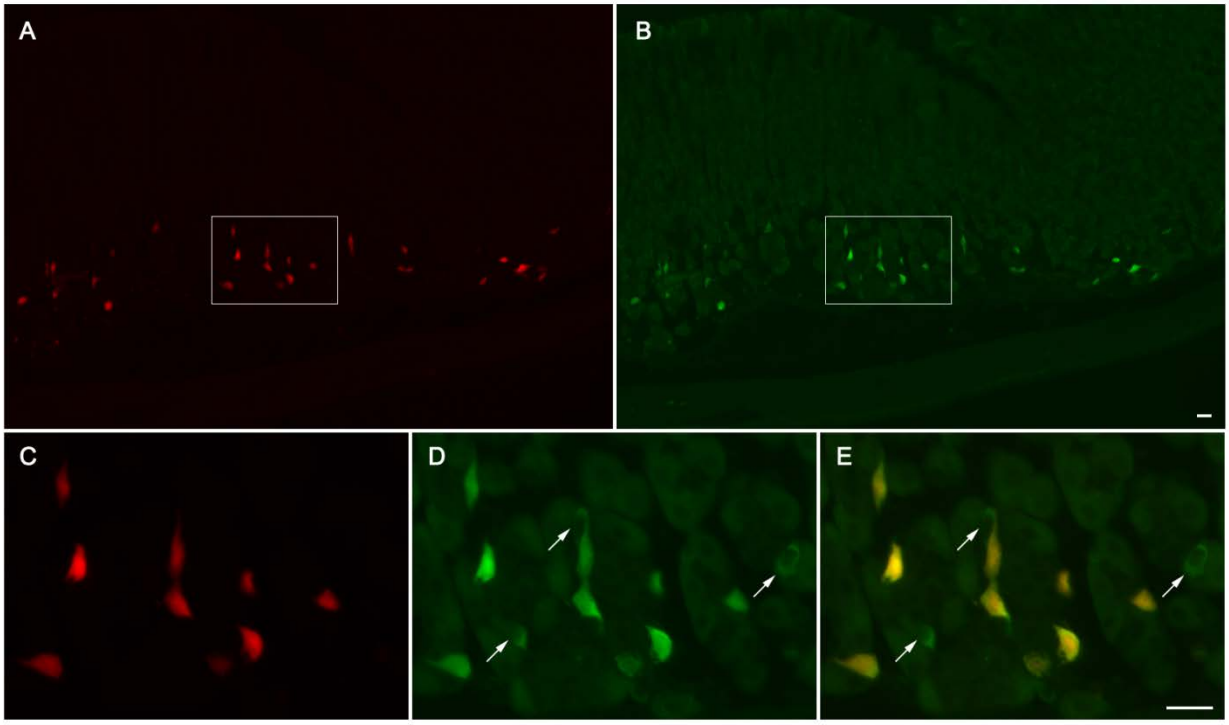

Figure 5.2. Co-localization of Tmt and HDC within the gastric oxyntic mucosa of HDC/Tmt mice. A–B) Low magnification photomicrographs showing the expression of Tmt fluorescence (A) within HDC-immunoreactive cells (B); the optics used to take these photomicrographs were such that only the elongated cells are visualized, and not the oval/fried egg-shaped cells or tiny round cells. C–E) High magnification views demonstrate that most (or in this region, all) cells with HDC-immunoreactivity (D) also express Tmt fluorescence (C). Arrows in (D) and in the overlay (E) indicate representative HDC-immunoreactive cells that lack Tmt fluorescence. Scale bar in (B) is 20 µm and also applies to (A). Scale bar in (E) is 20 µm and also applies to panels (C) and (D).

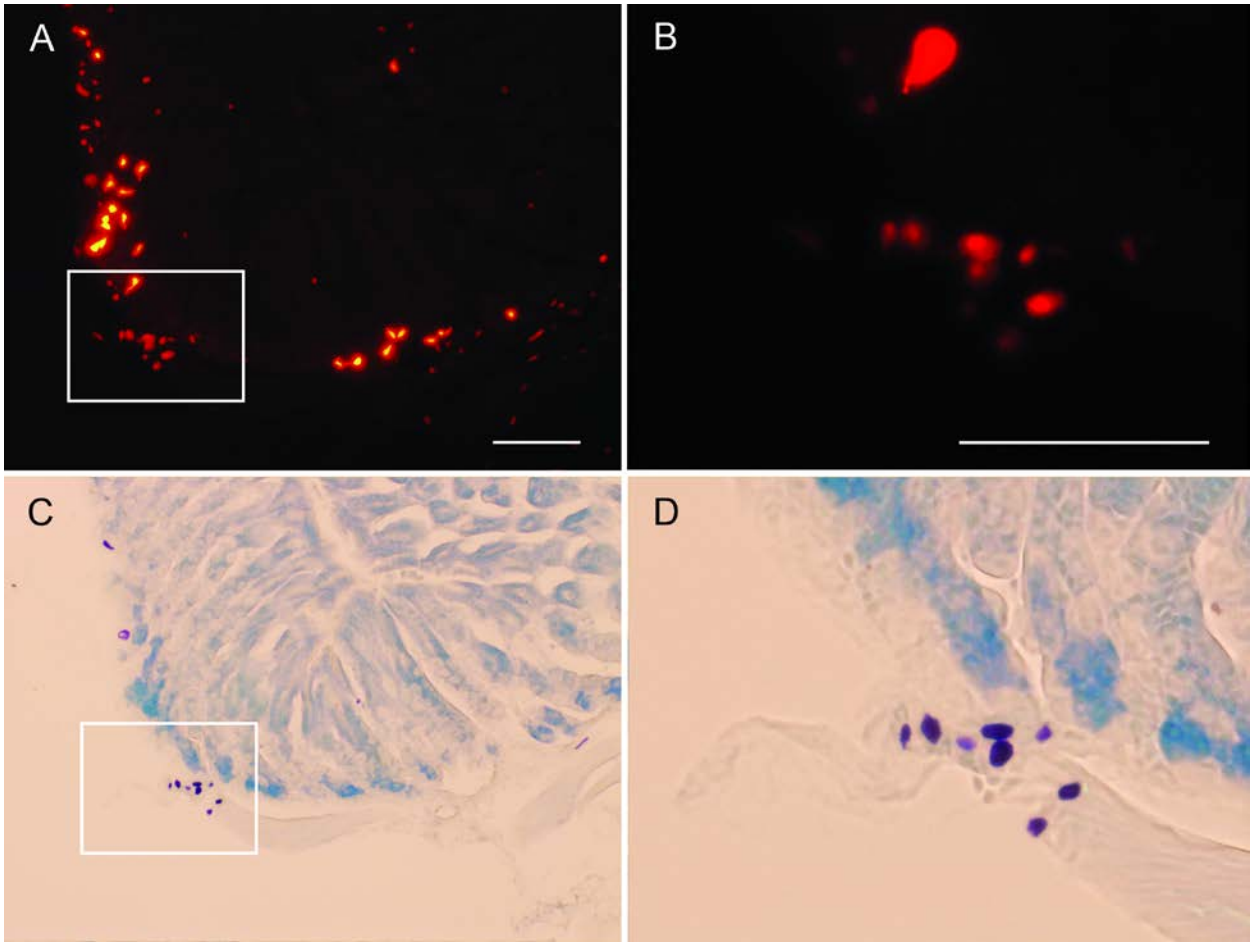

Figure 5.3. Expression of Tmt within mast cells of the stomach. A–B) Expression of Tmt fluorescence within the HDC/Tmt mouse stomach. C–D) Exclusive expression of toluidine blue stain within those HDC-immunoreactive cells with an oval/fried egg shape. Scale bar in (A) is 100 µm and also applies to (C). Scale bar in (B) is 100 µm and also applies to (D).

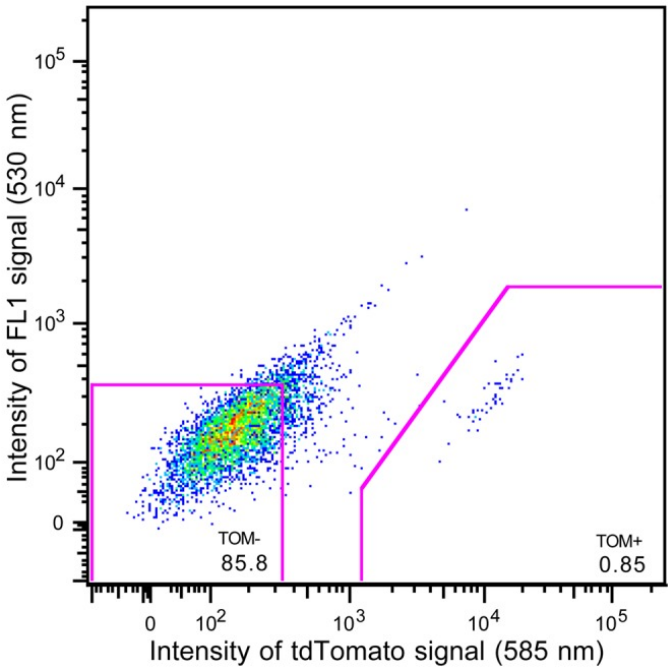

Figure 5.4. Fluorescence activated cell sorting of gastric mucosal cells. Graphical representation of FACS of mucosal cells from one representative set of HDC/Tmt mice, indicating cells collected as part of the Tmt-enriched pool (0.85% of the total number of sorted, living cells) and those collected as part of the Tmt-negative pool (85.8% of the total number of sorted, living cells).

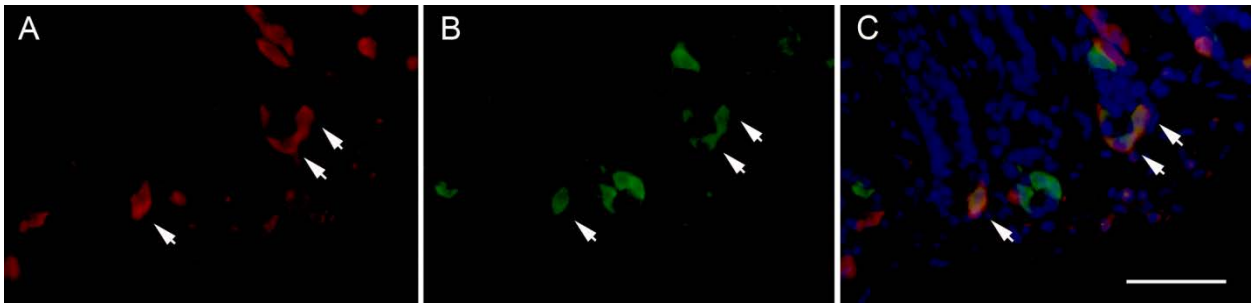

Figure 5.5. Co-localization of HDC with gastrin by dual-label immunofluorescence within the distal gastric mucosa. A few HDC-immunoreactive cells (B) co-express gastrin-immunoreactivity (A), and vice versa, as indicated by arrowheads as well as the yellow-orange color in the overlay (C). Blue color represents DAPI nuclear stain (C). Scale bar is 50 μm and applies to all panels

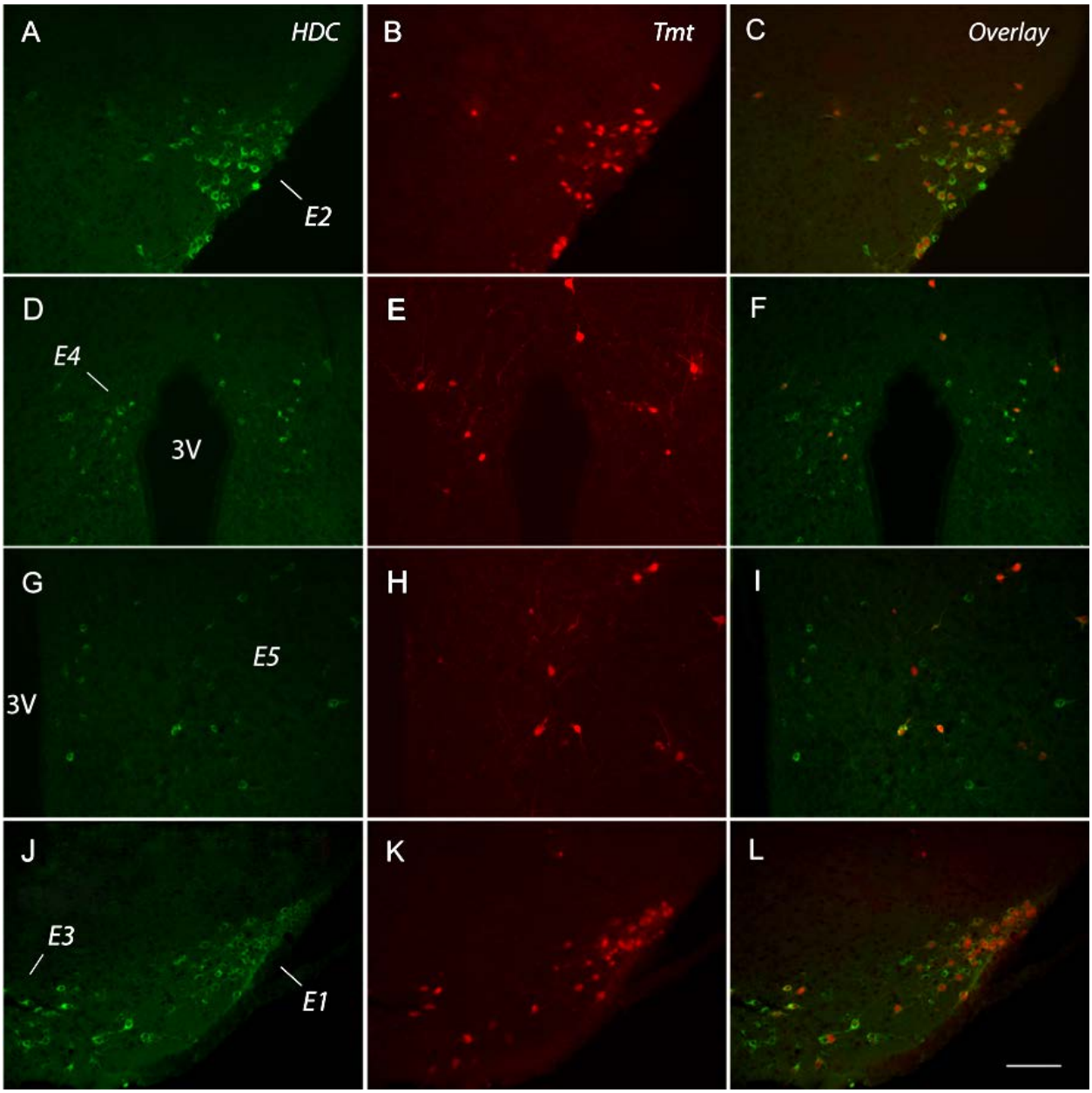

Figure 5.6. Expression of Tmt and HDC within the tuberomamillary nucleus histaminergic regions E1–E5 in HDC/Tmt mice. A,D,G,J) HDC-immunoreactive cells of representative sections for each histaminergic region. B,E,H,K) Tmt fluorescence exhibited in the same representative sections as shown in the left column. C,F,I,L) Overlay of the two images representing the co-localization of HDC immunoreactivity and Tmt fluorescence. Scale bar is 50 µm and applies to all panels. (3V – 3rd ventricle; in each column, sections arranged from the top to bottom as more rostral to more caudal).

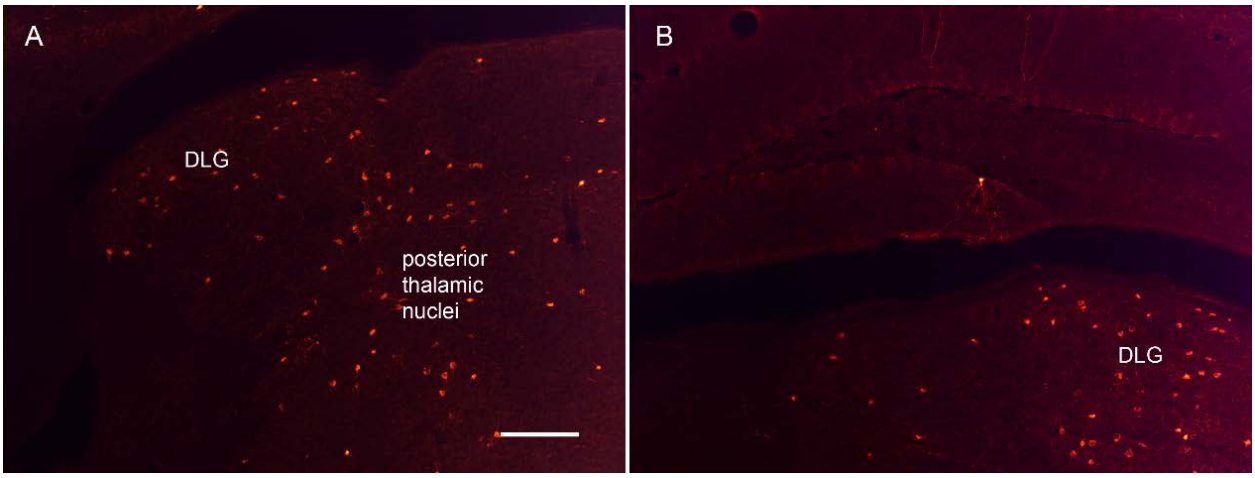

Figure 5.7. Non-specific expression of Tmt in the brain. A–B) Photomicrographs depicting presumed non-specific Tmt fluorescence (in cells without HDC-immunoreactivity) in the dorsal lateral geniculate nucleus (DLG), posterior thalamic nuclei, and hippocampus. Scale bar is100 µm and applies to both panels.

	% of cells with Tmt fluorescence co-expressing HDC-immunoreactivity	% of HDC immunoreactive cells co-expressing Tmt fluorescence
gastric mucosa	94.5±1.4	73.4±2.0

Table 5.1. Co-expression of Tmt and HDC within gastric body mucosa of HDC/Tmt mice. The data are reported as the mean percentage ± SEM for the gastric mucosa in the stomach at 4 different planes with 5 fields counted per plane; each plane is separated by at least 70 µm (n = 5 mice).

	Mucosa	Tmt-enriched pools	Tmt-negative pools
tomato (Tmt)	undetermined#	61.74±14.09***	*1.0±0.23 (30.82±0.23)*
histidine decarboxylase (HDC)	1.0±0.38 (23.91±0.35)	25.44±2.15***	0.08±0.03
PAC1	1.0±0.28 (23.81±0.33)	3.91±1.07*	0.14±0.06
tryptase beta-2 (Tpsb2)	1.0±0.62 (24.15±.80)	619.71±239.22*	4.06±2.81
chromagranin A	1.0±0.37 (18.42±0.33)	5.57±1.45*	0.63±0.42
prohormone convertase 1/3	1.0±0.43 (22.73±0.36)	5.85±1.89*	1.37±0.49
prohormone convertase 2	1.0±0.20 (22.73±0.17)	3.13±0.79*	0.90±0.13
calpain 8	1.0±0.11 (20.05±0.12)	0.18±0.14	0.48±0.07
H+/K+ ATPase β-subunit	1.0±0.13 (16.18±0.28)	0.25±0.13	0.90±0.34
GIF	1.0±0.27 (17.59±0.29)	0.27±0.08	1.34±0.28
pepsinogen F	1.0±0.12 (20.96±0.18)	0.59±0.32	0.80±0.19
ghrelin	1.0±0.21 (15.95±0.20)	0.29±0.06	0.90±0.14
somatostatin	1.0±0.30 (19.24±0.32)	0.33±0.18	0.59±0.26
tryptophan hydroxylase	1.0±0.63 (24.44±1.0)	649.16±172.47***	18.76±1.20
gastrin	1.0±0.42 (21.11±0.62)	643.25±304.92*	1.11±0.20

Table 5.2. Relative mRNA expression levels of various mRNAs in gastric mucosa and FACS-separated pools of gastric mucosal cell populations. All values are normalized to the

housekeeping gene cyclophilin. With the exception of Tmt, each value represents the amount of mRNA relative to that within gastric mucosa preparations from C57BL6/J mice.

[#]The mRNA level was lower than the detection limit; because Tmt is not found in (wild-type) C57BL6/J mice, the value of Tmt in the Tmt-enriched pools represents the amount of mRNA relative to that of Tmt-negative pools.

*P<0.05, ***P<0.001 as compared to mucosa (or in the case of Tmt, as compared to Tmt-negative pools) when determined by Dunnett's post-hoc analysis.

Values in parenthesis denote the mean ± SEM of threshold cycles. (n = 3–4 FACS preparations/gene).

	% of cells with HDC-immunoreactivity co-expressing gastrin-immunoreactivity	% of cells with gastrin-immunoreactivity co-expressing HDC-immunoreactivity
gastric antrum	29.1±2.1	17.3±1.7

Table 5.3. Co-expression of HDC and gastrin within the gastric antrum mucosa of mice.

The data are reported as the mean percentage ± SEM for the gastric antrum in the stomach at 8 different planes with 5 fields counted per plane; each plane is separated by at least 70 µm (n = 3 mice).

Histaminergic Brain Region	% of cells with Tmt fluorescence co-expressing HDC-immunoreactivity	% of HDC immunoreactive cells co-expressing Tmt fluorescence
E1	91.6±3.6	80.8±3.8
E2	97.5±1.0	81.8±4.0
E3	90.6±4.2	82.1±4.2
E4	92.3±2.7	54.9±5.7
E5	91.1±3.7	43.2±3.3

Table 5.4. Co-expression of Tmt and HDC within the tuberomamillary nucleus of HDC/Tmt mice.

The data are reported as the mean percentage ± SEM for histaminergic brain regions E1–E5, each section separated by 80 µm; every section containing histaminergic regions in the hypothalamus was counted (n = 3 mice).

DISCUSSION

My thesis work covers an expanse range of topics that intersect through a common theme: ghrelin. In Chapter 2, I investigated how the loss of ghrelin signaling affects DG neurogenesis during stress and correlated these changes in neurogenesis with ghrelin's effect on depressive-like behavior. I also assessed whether the severity of depressive-like behavior could be reduced by protecting DG neurogenesis during stress. As my most elaborate piece of work, this chapter features the use of P7C3-related compounds, which may have therapeutic potential for mood-related disorders. Chapter 3 discusses my studies demonstrating a role for ghrelin in the cue-potentiated feeding response. Moving on from the behavioral aspects of ghrelin, Chapter 4 focuses on a cellular point of view, studying ghrelin cell physiology and ghrelin cell synthesis/secretion of potential proteins cell other than ghrelin. Lastly, Chapter 5 describes my characterization of a novel mouse model for studies of the histaminergic system, which includes a histamine-producing enteroendocrine neighbor of the ghrelin cell- the ECL cell.

Chapter Two Conclusions: *The P7C3-class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis*

Chapter two is a compilation of my most extensive work (Walker et al., 2014). Stemming from a desire to further understand the association between ghrelin, hippocampal neurogenesis, and mood regulation, this study provides strong evidence for anti-depressant mechanisms involving DG neuroproliferation and neuroprotection. Previous studies from the Zigman lab determined that after CSDS, *Ghsr*-null mice display more severe depressive-like behavior then wild-type littermates. After uncovering a wealth of literature on ghrelin's neuroprotective and neuroproliferative characteristics, I hypothesized that perhaps the increase of depressive-like behavior in stressed *Ghsr*-null mice reflected a more severe reduction of neurogenesis in

response to stress. Prior to this study, the role of stress-induced elevations in ghrelin on hippocampal neurogenesis and the resulting effect on depressive-like behavior was not thoroughly evaluated. Analysis of DG proliferation and cell survival revealed that ghrelin protects against a greater loss of DG ventral neurogenesis with stress, whereas there was no significant effect of stress-induced ghrelin in the dorsal DG. This matched previous studies in which chronic unpredictable stress more negatively impacted the ventral rather than the dorsal DG (Hawley, D.F. et al. 2012; Hawley, D.F. et al. 2012). While these regional effects may seem curious, ghrelin receptor expression was found to be higher in the ventral DG as compared to the dorsal DG, suggesting that the two regions may exhibit different expression levels for many receptors involved in the stress response.

The severity of ventral DG cell death and reduced proliferation in *Ghsr*-null mice corresponded with the increased severity of depressive-like behavior, hinting that neurogenesis may be involved. It was suspected that protecting against stress-induced reductions of neurogenesis would also protect against the development of a more severe depressive phenotype. Administration of anti-apoptotic, P7C3-related compounds indeed blocked neurogenic loss and attenuated depressive-like behavior in mice, although only the stronger P7C3-A20 compound affected wild-type mice. Importantly, ablation of neurogenesis inhibited the anti-depressant efficacy of P7C3-A20.

Broadening the study, an additional measure of anti-depressant behavior was utilized in determining P7C3's effect on mood. Previously, it has been shown that caloric restriction has an anti-depressant effect by reducing the time spent immobile in the FST for wild-type mice, but not *Ghsr*-null mice (Lutter, M. et al. 2008). In my current study, P7C3 rescued this anti-depressant response to caloric restriction in *Ghsr*-null mice, suggesting that caloric restriction mediates molecular signals that function through DG neurogenesis in order to produce the anti-

depressant response. Pertaining to this, caloric restriction increased cell survival in the DG of wild-type mice while decreasing survival in *Ghsr*-null mice; the lowered cell survival was normalized with P7C3 administration.

Chapter Two Significance

For this study, I utilized the 10-day CSDS protocol as a model of chronic psychosocial stress featuring aspects of major depression and posttraumatic stress disorder. This behavioral model has been used repeatedly by several groups and consistently produces a depressive phenotype in a significant portion of C57Bl/6J mice (Berton, O. et al. 2006; Lutter, M. et al. 2008; Lagace, D.C. et al. 2010; Christoffel, D.J. et al. 2011; Chuang, J.C. et al. 2011; Golden, S.A. et al. 2011; Kumar, J. et al. 2013; Donahue, R.J. et al. 2014). The CSDS paradigm has been thoroughly assessed as a reliable model of major depression by the Nestler group (Berton, O. et al. 2006; Krishnan, V. et al. 2007). In one particular study, extensive behavioral testing was performed after exposure of C57Bl/6J mice to CSDS using various measures of depressive-like behavior (Krishnan, V. et al. 2007). Tests such as the SI test, FST, tail suspension test (TST), and sucrose preference tests were conducted to gauge relative levels of social avoidance, despair, and anhedonia (Krishnan, V. et al. 2007). Alternative procedures were also performed after CSDS, including measurements of locomotor activity, anxiety, body weight, cardiac hypertrophy, circadian amplitude, cocaine CPP, social hyperthermia, stress-induced polydipsia, and corticosterone levels (Krishnan, V. et al. 2007). Mice that displayed depressive-behavior according to the SI test were termed “susceptible,” while the others were categorized as “unsusceptible.” The susceptible mice differed from unsusceptible mice by displaying increased anhedonia (decreased sucrose preference), increased cocaine-CPP, increased stress-induced polydipsia, increased social hyperthermia, reduced circadian amplitude, and weight loss (Krishnan, V. et al. 2007). Since the degree of social avoidance in

the SI test correlates with the appearance of these additional depression-related symptoms, CSDS is a strong model for studies related to depression. CSDS-induced social avoidance behavior is persistent and has been shown to last up to 39 days after the last day of CSDS, unlike other depressive measures (Krishnan, V. et al. 2007). Studies have also been performed to monitor CSDS-induced changes in self-grooming, food intake, meal patterns, CPP for HFD, and lipid synthesis (Chuang, J.C. et al. 2010; Denmark, A. et al. 2010; Chuang, J.C. et al. 2011; Kumar, J. et al. 2013), revealing disturbances consistent with depression. Chronic administration of fluoxetine or imipramine following the completion of CSDS resulted in a reversal of social avoidance behavior, again suggesting the validity of this model as a measure of depressive-behavior (Berton, O. et al. 2006; Tsankova, N.M. et al. 2006). Thus, while social avoidance may not capture all the facets of depressive-like behavior in humans, I do feel it is a sufficient behavioral correlate of human depression that provides important lessons regarding the mechanisms of human depression and efficacy of putative therapeutic drugs.

The proneurogenic, anti-apoptotic effects of P7C3-related compounds remain the only functional observations of this compound at the cellular level. The unique idea to test the anti-depressant efficacy of a compound that was specifically discovered for its neuroprotective properties truly sets this study apart from others. Although many of the currently marketed anti-depressants also enhance neurogenesis, they are not primarily characterized by their proneurogenic properties as P7C3 is (Pieper, A.A. et al. 2010; MacMillan, K.S. et al. 2011; De Jesus-Cortes, H. et al. 2012; Tesla, R. et al. 2012; Blaya, M.O. et al. 2014). Furthermore, the degree and rapidity in which P7C3-related compounds elevate the proliferating DG population is unmatched by other anti-depressants. In sum, the therapeutic benefit of enhancing or protecting DG neurogenesis in individuals suffering from depression is quite promising, particularly in those exposed to high stress environments. These findings also allude to the

possible advantage of proneurogenic compounds in preventative treatments for those with great risk of developing depression related to post-traumatic stress disorder, such as soldiers.

The use of *Ghsr*-null mice in these mood related studies emphasizes the association between metabolic syndromes and psychiatric disorders such as depression. It is important not to overlook this relationship, since manipulations in ghrelin signaling can have a direct impact on feeding and body weight regulation as well as mood. While ghrelin may have anti-depressant properties, its elevation can lead to increased feeding and weight gain, which also occurs with many marketed antidepressants (Berken, G.H. et al. 1984; Russ, M.J. et al. 1988; Fava, M. 2000; Hasnain, M. et al. 2012). Therefore, the discovery of novel anti-depressants that do not negatively affect body composition or contribute to unwanted weight gain may result in less aversive treatments for patients. Without this negative side effect, individuals suffering from depression may be more consistent in taking their medication, which is crucial for proper control of the disease. Of note, P7C3 administration did not significantly affect body weight in my study, although extended studies are needed to verify potential long term effects.

Moreover, due to their enhanced sensitivity to the harmful effects of chronic stress, both in terms of depression and neurogenesis, *Ghsr*-null mice were quite advantageous in studying P7C3 anti-depressant potential. The severe degree of stress-induced depressive-like behavior and neurogenic loss in these mice allowed P7C3-related compounds to have a greater, more obvious impact, both in terms of cell rescue and attenuation of depression. The robust anti-depressant effect in *Ghsr*-null mice has many implications in terms of human therapeutic benefits. Particularly, individuals with depression related to dysfunctional ghrelin signaling may benefit from treatment with neuroprotective agents. In fact, reductions in plasma ghrelin after Roux-en-Y gastric bypass surgery has been hypothesized to contribute to the increased suicide risk in bariatric surgery patients (Mitchell, J.E. et al. 2013; Nannipieri, M. et al. 2013; Malin, S.K.

et al. 2014). Even at 1 year post-surgery, lowered ghrelin levels have been observed in Roux-en-Y gastric bypass patients, making it conceivable that depression associated with bariatric surgery precipitates from disturbances in neurogenesis (Nannipieri, M. et al. 2013).

Another substantial observation from my study is P7C3's reliance on intact neurogenesis in order to demonstrate anti-depressant efficacy. A handful of studies have shown that ablation of neurogenesis also blocks the antidepressant efficacy of certain retailed antidepressants, although findings from other studies are inconsistent with this notion. The discrepancies between these studies seem to be related to differences in behavioral tests, presence/absence of stress, methods of stress, ablation techniques, length and timing of study, and antidepressant administered, among others (Santarelli, L. et al. 2003; Jiang, W. et al. 2005; Meshi, D. et al. 2006; Surget, A. et al. 2008; Wang, J.W. et al. 2008; Bessa, J.M. et al. 2009; David, D.J. et al. 2009; Zhu, X.H. et al. 2010).

I would like to point out that P7C3 administration did not have an anti-depressant effect in control, ad-libitum fed mice. This supports the idea that antidepressant aspects of neurogenesis may come from a normalization of levels rather than increasing neurogenesis beyond basal levels (Petrik, D. et al. 2012). Likewise, in stressed or calorically restricted *Ghsr*-null mice, P7C3 administration normalized DG cell death to baseline levels and resulted in anti-depressant behavior. It seems that the anti-depressant effects of caloric restriction are dependent on a basal level of neurogenesis in order to develop, suggesting that molecular signals induced by caloric restriction function through a required level DG neurogenesis to prompt anti-depressive behavior. Similarly, in the setting of stress, severe reductions from basal levels of neurogenesis seem to sensitize *Ghsr*-null mice to the depressive effects of chronic stress. Restoration of basal neurogenesis with P7C3-related compounds protects these mice from acquiring a more severe depressive phenotype.

The distinction between dorsal and ventral DG in my study adds to a plethora of evidence suggesting a functional dissociation between dorsal and ventral regions of the hippocampus. Mapping out neuronal pathways of the hippocampus, it was discovered that the major cortical connections to the entorhinal cortex (EC) and projections to the DG could be divided into three zones (Witter, M.P. et al. 1989; Dolorfo, C.L. et al. 1998; Dolorfo, C.L. et al. 1998). These three zones project in a topographic manner to distinct regions along the DG, consequently forming septal/dorsal, intermediate, and temporal/ventral regions of the DG (Ruth, R.E. et al. 1982; Dolorfo, C.L. et al. 1998; Dolorfo, C.L. et al. 1998; Moser, M.B. et al. 1998). While there is a degree of overlap in the distribution of associational fibers between the septal and intermediate DG regions, temporal DG associational fibers are mostly segregated from the other two regions (Moser, M.B. et al. 1998). Projections from the hippocampus are also separated as the dorsal two-thirds of the subiculum, the output of the hippocampal formation, projects to the mammillary complex while the ventral subiculum connects to the amygdala, NAc, and rostral hypothalamus (Krettek, J.E. et al. 1977; Swanson, L.W. et al. 1977; Canteras, N.S. et al. 1992). Altogether, the neuronal connectivity of the hippocampus suggests that dorsal and ventral segments of the longitudinal hippocampal axis differ in terms of input and output. Moreover, the ventral hippocampus is more dense with dopaminergic, noradrenergic, and serotonergic terminals (Gage, F.H. et al. 1980; Verney, C. et al. 1985).

Researchers began to question whether these neurochemical and structural differences revealed distinct functional outputs for the dorsal and ventral hippocampus. One initial study revealed that the dorsal 70% of the hippocampus was required to be intact for spatial memory retrieval, but not the ventral hippocampus (Moser, M.B. et al. 1998). In addition, while rats with ventral lesions were unaffected in learning delayed alternation in T-maze tasks, they did show impairments in learning internal state-shock associations, which is associated which emotional

learning (Hock, B.J., Jr. et al. 1998). More recent studies have exhibited that intact dorsal hippocampus is necessary for the learning and expression of spatial discrimination tasks (Chai, S.C. et al. 2004; Gaskin, S. et al. 2006; White, N.M. et al. 2006). Gene expression in the dorsal hippocampus was found to correlate with information processing cortical brain regions, while regions involved in processing emotion or stress exhibited gene expression closely related to the ventral hippocampus (Fanselow, M.S. et al. 2010). The intriguing possibility that adult neurogenesis in the dorsal and ventral DG may be functionally dissociated has been supported by correlational studies. In humans, selective serotonin reuptake inhibitors and tricyclic antidepressants elevate proliferating neuronal precursors more in the anterior DG, which corresponds to the ventral DG in rodents (Boldrini, M. et al. 2009). The antidepressant agomelatine increased neurogenesis selectively in the ventral DG, leading authors to hypothesize that there may regional distinctions in the DG's regulation of mood. In sum, it seems the dorsal hippocampus (posterior hippocampus in primates) primarily influences cognitive function, and the ventral hippocampus (anterior hippocampus in primates) shapes behaviors related to stress, emotion, or affect (Fanselow, M.S. et al. 2010).

Lastly, the beneficial effects of P7C3 pertained only to depressive-like behaviors and had no effect on anxiety. In emphasizing this specificity, I have shown that the neurochemical pathways involved in the etiology of depression may be distinct from those involved in anxiety. Since the effectiveness of P7C3 on reducing depression was not paralleled with a reduction in anxiety, there is clearly some divergence in the mediation of these psychiatric disorders. Still, the pathways likely intersect at some points, and there may be overlapping molecular mediators involved in the precipitation of both depression and anxiety.

Chapter Two Future Studies

In general, the etiology of depression remains mostly a mystery. Consequently, it is impractical to predict which individuals will definitely develop a depressive-disorder, so preventative medications may not be applicable to many who already suffer. Future studies involve manipulating the timing of P7C3-A20 administration in the CSDS model. While my study reveals that P7C3-related compounds *prevent* the increased severity of stress-induced depression, I would like to assess whether it can also diminish existing depressive behavior in mice. Hence, mice would not be administered P7C3-A20 until after verifying CSDS-induced depressive behavior. Rather than administering the compound during CSDS, mice would be treated chronically with P7C3-A20 for different lengths of time after CSDS and the SI test. Successful antidepressant efficacy in this paradigm would divulge even greater potential for P7C3-related compounds in treating individuals currently experiencing depression.

Stress-induced elevations in ghrelin not only influence depression, but also food reward behaviors. An innovative previous study revealed that CPP for high fat diet is precipitated by chronic stress in wild-type mice, but not *Ghsr*-null mice (Chuang, J.C. et al. 2011). Considering the severity of stress-induced depressive behavior and DG neurogenic loss in *Ghsr*-null mice, this may reflect an increased state of anhedonia or a learning dysfunction. There is also likely a strong food reward component to this behavior, and the VTA was found to partially mediate ghrelin's effects on CPP demonstration (Chuang, J.C. et al. 2011). Forthcoming studies will determine whether DG neurogenesis is involved in the development of stress-induced CPP for high fat diet. Utilizing P7C3-A20 administration during stress to protect neurogenesis, the rescue of CPP for high fat diet in *Ghsr*-null mice can be attempted.

The dissection of the dorsal and ventral DG functionality is imminent in future studies. The *Ghsr*-null mouse, in which a transcriptional blocker flanked by loxP sites was strategically

placed in front of the *Ghsr* gene, is a valuable tool for targeting regional or cell-specific reexpression of ghrelin receptors (Chuang, J.C. et al. 2011). In an attempt to distinguish the role of dorsal versus ventral ghrelin signaling in stress-induced depressive-behavior, microinjection of an associo-adenovirus expressing Cre-recombinase (AAV-Cre) can be performed, directed at each specific subregion. If ghrelin signaling in the ventral DG is fundamental for mood regulation, then *Ghsr* expression solely in the ventral DG should be sufficient in diminishing the severity of depressive-like behavior.

Differentiating the transcriptional and molecular profiles of the dorsal and ventral DG in response to stress will also aide our understanding of how these regions affect depression. I have demonstrated that *Ghsr* expression is elevated in the ventral DG as compared to the dorsal DG in naiive, non-stressed mice. However, chronic stress can result in transcriptional and/or translational changes in the DG. In fact, the DG is particularly susceptible to the stress response due to an abundance of glucocorticoid receptor expression (Reul, J.M. et al. 1985), and persistently elevated corticosterone levels that occur with chronic stress have a major impact on DG neurogenesis and cell survival (Wong, E.Y. et al. 2004; Yu, I.T. et al. 2004; Robertson, D.A. et al. 2005; Joels, M. et al. 2007; Brummelte, S. et al. 2010). Scrutinizing differences in the dorsal and ventral DG, one study found that the distribution of glucocorticoid receptors in the dorsal versus ventral DG fluctuates unevenly in response to different lengths of stress (Robertson, D.A. et al. 2005). Therefore, it would be exciting to examine whether stress-induced changes in DG glucocorticoid receptor expression differs in *Ghsr*-null mice as compared to wild-type littermates, as ghrelin signaling may affect receptor expression. I would also explore the possibility of differential *Ghsr* expression in the dorsal versus ventral DG in response to stress.

Alternatively, DG neurogenesis heavily influences hippocampal negative feedback of the HPA axis (Snyder, J.S. et al. 2011). One clever study recognized that DG neurogenesis mitigates the stress response, allowing faster glucocorticoid recovery in response to stressful stimuli (Snyder, J.S. et al. 2011). In other words, DG neurogenesis acts to prevent a chronic hyperelevation in glucocorticoid levels in response to stress. Inspired by this observation, one future study involves monitoring corticosterone levels in stressed *Ghsr*-null and wild-type mice, with or without P7C3-A20 administration. I hypothesize that the protection of DG neurogenesis conferred by P7C3-A20 will result in a quicker recovery of stress-induced elevations in corticosterone. This strong negative feedback on glucocorticoid secretion may very well contribute additional means of protection against stress-induced depression, especially since chronic glucocorticoid elevations are associated with mood disorders (Joels, M. 2011; Lehmann, M.L. et al. 2013).

A critical detail that remains to be uncovered is the specific target of P7C3-related compounds. This vital piece of information may reveal additional pathways or functions for P7C3 which could have major effects on the interpretation of my study. Presently, it is believed that P7C3 protects cellular distress in toxic environments by stabilizing the mitochondrial membrane (Pieper, A.A. et al. 2010). However, a molecular target with which P7C3 directly interacts or binds to has yet to be identified.

In the end, the goal of the study was to link the severity of depressive-like behavior to the severity of reduced neurogenesis by pharmacologically rescuing neurogenesis, and in doing so, discovering a potential new target and method for therapeutic treatment of severe depression. Reducing the depressive-like behavior of *Ghsr*-null mice by rescuing cell death and increasing the number of mature neurons not only supports the assertion that regulation of

neurogenesis can impact mood, but it also provides a mechanistic explanation for ghrelin's protective effects on mood after stress.

Chapter Three Conclusions: *Disruption of cue-potentiated feeding in mice with blocked ghrelin signaling*

The central theme of this study (Walker, A.K. et al. 2012) encompasses ghrelin's involvement in the normal development of a cue-potentiated feeding response. A novel cue-potentiated feeding protocol was designed for this study. Employing this new method, food-sated C57BL/6J mice increased consumption of grain-based pellets after presentation of a conditioned stimulus previously paired with these pellets during caloric restriction. Successful demonstration of the cue-potentiated feeding response in C57BL/6J mice allowed me to next determine whether ghrelin signaling plays a part in this behavior.

Indeed, I discerned that proper ghrelin signaling is necessary in order to develop a normal cue-potentiated feeding response. Blocking ghrelin signaling with a GHSR antagonist during conditioning sessions later prevented a potentiation of food intake when presented with a positive cue. Genetic disruption of ghrelin signaling in *Ghsr*-null mice led to the potentiation food intake in response to both a negative and positive cue, suggesting a misassociation of the negative cue with food. Furthermore, *Ghsr* expression was detected in the BLA, and neuronal activation in this region corresponded with the amount of pellets eaten in the cue-potentiated feeding response. Thus, ghrelin's control over the development of proper cue-based potentiation of feeding is now hypothesized to operate partly through the BLA.

Chapter Three Significance

With the evolution of advertising, logos representing commercial food products are abundant in our everyday environment including the internet, television, billboards, magazines, and other media sources (Halford, J.C. et al. 2004; Cohen, D.A. 2008). Incredibly, a study performed in 2007 collected data from food commercials appearing during top-rated television shows and discovered that 97.8% of the advertisements viewed by children were high in fat, sugar, or sodium (Powell, L.M. et al. 2007). Sugar comprised 46.1% and 49.1% of the total calories among advertised products seen by children and adolescents, respectively (Powell, L.M. et al. 2007). In 2011, it was observed that children's exposure to fast-food advertising was trending upward with a 30.8% peak increase (Powell, L.M. et al. 2011). A more recent study from 2013 revealed that about 95.8% to 97.3% of all the ads seen on children's programming were for products high in recommended nutrients to limit, including saturated fat, trans fat, sugar, and sodium (Powell, L.M. et al. 2013).

With recurring exposure to these cues, children and adults are at risk of strengthening neurochemical pathways that associate these cues with the pleasurable aspect of eating (Holland, P.C. et al. 2005). These cue-food associations are powerful enough to override satiety signals and instigate habitual eating which can contribute to the development of obesity (Holland, P.C. et al. 2005). Consequently, a thorough understanding of the molecular pathways, signaling proteins, and brain regions involved in the foundation and exhibition of cue-potentiated feeding is critical.

The creation of a novel cue-potentiated feeding protocol is significant because it allowed the classic cue-potentiated feeding studies to be replicated in mice (Holland, P.C. et al. 2005; Petrovich, G.D. et al. 2005). While this behavioral paradigm has been executed consistently and repeatedly in rats, prior to this study, it had not been performed in mice using a non-savory food.

This use of non-savory food is a vital detail since the involvement of tasty or pleasurable food may obscure the pathways necessary for simple cue-food association by introducing a stronger component of reward. Replicating this study with sucrose pellets is a noteworthy idea, as the results between the two studies could be compared side-by-side to determine how the added dimension of a rewarding food might change the cue-potentiated feeding response.

The requisite for ghrelin in the cue-potentiated feeding response is substantial because it provides possible methods for lessening the risk of developing of a pervasive cue-induced feeding response. For example, perhaps watching television right before mealtimes increases the chance of acquiring cue-food associations since ghrelin levels peak before meals. Additionally, stress-induced elevations in ghrelin may contribute to a stronger cue-potentiated feeding response as well, so the importance of managing stress levels is crucial as stress may largely impact eating habits. Ghrelin's mediation of cue-potentiated feeding also exposes ghrelin as potential target for treatment in addictive eating behaviors, although the roles of ghrelin are diverse and may complicate obtaining specificity in behavioral treatments. Therefore, dissecting which brain regions ghrelin is acting upon to influence various behaviors, such as cue-potentiated feeding, will facilitate the targeting of specific ghrelin-induced behavioral effects in the future.

Pinpointing *Ghsr* expression specifically in the BLA is attractive in this study because connections from the BLA to the mPFC are obligatory for the development of basic cue-potentiated feeding (Petrovich, G.D. et al. 2005). I have also shown that the amount of BLA activation in response to a positive cue corresponds with the amount of food eaten. Yet, *Ghsr*-null mice experienced BLA activation and potentiated food intake in response to both the negative and positive cue, revealing their inability to discriminate between the cues when forming an association to food. This representation of improper learning expands upon what is known in the current literature to signify ghrelin's participation in learning and memory-related

processes. In fact, ghrelin-KO mice perform poorly in novel object recognition tests and have deficits in contextual fear conditioning, which is mediated by the amygdala (Goosens, K.A. et al. 2001; Diano, S. et al. 2006; Albarran-Zeckler, R.G. et al. 2012). Memory retention was dose-dependently enhanced by direct microinjection of ghrelin into the amygdala or hippocampus (Carlini, V.P. et al. 2004). Similarly, microinjection of ghrelin into the ventral hippocampus heightened cue-potentiated feeding responses in rats (Kanoski, S.E. et al. 2013) Taken together, ghrelin augments cue-related learning and memory, while the disruption of ghrelin signaling interferes with the recognition and memory of distinct objects or cues. Given ghrelin's actions in the hippocampus and amygdala and the strong learning component in cue-based learning, it is likely that these two brain regions are mainly responsible for ghrelin's effects on cue-potentiated feeding.

Chapter Three Future Directions

My studies of cue-potentiated feeding in mice allude to ghrelin action in the BLA for normal development of this feeding behavior. However, the genetic and pharmacologic manipulations on ghrelin signaling (GHSR antagonist, *Ghsr*-null mice) were systemic rather than directly targeting the BLA. Direction microinjection of ghrelin into the BLA during conditioning sessions and/or during the test day of the cue-potentiated feeding protocol would distinguish whether ghrelin signaling in the BLA is sufficient to induce a heightened cue-based feeding response. Extending upon this idea, re-expression of *Ghsr* in the BLA of *Ghsr*-null mice could be performed in an attempt to rescue the specificity of positive cue-food associations. This would be accomplished by microinjection of AAV-Cre into the BLA, which would remove the transcriptional blocker flanked by loxP sites in front of the *Ghsr* gene, allowing reexpression of *Ghsr* mRNA. In the instance that *Ghsr* reexpression in the BLA does not rescue the learning

deficit, the hippocampus can then be targeted for reexpression, or both the hippocampus and the amygdala as ghrelin may require multiple sites of action.

Though I have focused on the hippocampus and BLA as chief mediators of ghrelin's action on cue-potentiated feeding, it is reasonable that the VTA may share responsibility in the occurrence of this habitual behavior as well. The induction of feeding and increased consumption may be driven by cravings resulting from the cue, which relates to the reward system including the VTA. Used in a previous Zigman lab study, breeding TH-Cre mice with *Ghsr*-null mice results in reexpression of ghrelin receptors in catecholaminergic neurons, largely rescuing expression in the VTA (Chuang, J.C. et al. 2011). The utilization of these mice would unmask the relevance of the VTA in this food-related behavior. Although AgRP neurons primarily stimulate food intake to satisfy energy needs, they may also play a role in reward related behaviors (Hagan, M.M. et al. 2001; Olszewski, P. et al. 2003; Krashes, M.J. et al. 2011). Employing an innovative technique involving designer receptors exclusively activated by designer drugs (DREADD), specific and reversible AgRP neuronal activation was performed to determine the role of this population in feeding behavior (Krashes, M.J. et al. 2011). AgRP stimulation resulted in a rapid, potent feeding response while AgRP neuron inhibition reduced food intake (Krashes, M.J. et al. 2011). Strikingly, food seeking behavior and motivation to acquire food were both powerfully influenced by AgRP neuron activation, suggesting a role for AgRP neurons outside of homeostatic feeding regulation (Krashes, M.J. et al. 2011). Newly published data have revealed that projections from the PVN to AgRP neurons may mediate these behaviors (Krashes, M.J. et al. 2014). The Zigman lab has recently used an AgRP-CreER(T2) mouse model crossed with *Ghsr*-null mice in order to re-express ghrelin receptors in AgRP neurons after major brain development has occurred (Wang, Q. et al. 2014). These mouse models would also be useful tools in studying sites for ghrelin action in the development of cue-potentiated feeding.

Mechanistically, ghrelin's modulation of learning in the cue-potentiated feeding protocol may occur through synaptic plasticity. Ghrelin has been shown to enrich hippocampal spine density, increase synaptic plasticity, promote long term potentiation (LTP), and increase the incorporation of synaptic AMPA receptors (Diano, S. et al. 2006; Carlini, V.P. et al. 2010; Chen, L. et al. 2011; Ribeiro, L.F. et al. 2014). Ghrelin's effects on synaptic plasticity in the BLA remain unexplored, although a respectable amount of literature exists for behavioral effects of ghrelin in the amygdala (Toth, K. et al. 2009; Toth, K. et al. 2010; Alvarez-Crespo, M. et al. 2012; Goshadrou, F. et al. 2012; Song, L. et al. 2013). Investigating the effect of ghrelin on BLA spine density and synaptic plasticity in the cue-potentiated feeding paradigm would be a unique idea, introducing the concept of ghrelin and BLA plasticity. Hippocampal synaptic plasticity in response to cue-potentiated feeding could also be evaluated, including electrophysiological studies of LTP. These studies could be performed by comparing synaptic plasticity in *Ghsr*-null mice to that in wild-type littermates both in naiive, non-conditioned mice and in mice conditioned for cue-potentiated feeding.

To conclude, although proper ghrelin signaling is essential for the appropriate formation of cue-food associations, there is much to be learned about the cellular mechanisms and neural pathways involved. The importance of ghrelin signaling in the amygdala is only beginning to be delved into, and this study adds to the small collection of work springing up on this fresh idea.

Chapter Four Conclusions: *The expression of serum retinol binding protein and transthyretin within mouse gastric ghrelin cells*

The study performed here (Walker, A.K. et al. 2013) unveils the novel expression of two metabolically relevant proteins, RBP4 and TTR, in ghrelin cells. As compared to whole stomach, significant enrichments of RBP4 and TTR mRNA were observed in FAC-sorted ghrelin

cell pools as well as stomach and pancreatic ghrelinoma cell lines. While secretion studies revealed that RBP4 is present in both ghrelin cell lysate and cell culture medium, incubation with catecholamines or octanoic acid did not increase RBP4 secretion as occurs with ghrelin. Mice harboring ghrelinomas did not have a significant elevation in circulating RBP4, as also is observed with ghrelin. Moreover, transcriptional regulation of RBP4 was unaffected by caloric restriction, although TTR mRNA was reduced by both a 24 hour fast and a week-long 60% caloric restriction. The apparent divergence of ghrelin and RBP4 regulation reveals that there are likely different mechanisms and signaling pathways involved in the transcription and secretion of these proteins.

Chapter Four Significance

With advancing developments occurring in fields that study ghrelin's downstream effects on metabolic regulations, cognition, mental health, and other actions, it is imperative to understand the framework and components of ghrelin's source: the ghrelin cell. The physiology and protein expression profile of ghrelin cells remains highly unexplored, and the detailed signaling pathways that lead to hormone secretion are still under examination. In order to understand the ghrelin cell as a whole unit, it is important to understand how the molecular pieces work together to keep the cell smoothly running and participating in the network of gastric mucosal cells.

Identifying high RBP4 and TTR expression adds new weight to the biological significance of the ghrelin cell. As the only specific serum transport protein for retinol, RBP4 secretion from ghrelin cells may aide retinol-dependent functions such as growth and development or immune regulation (Graham, T.E. et al. 2007; D'Ambrosio, D.N. et al. 2011; Kotnik, P. et al. 2011). Although the liver contains the highest expression of RBP4 which is believed to contribute mainly to retinol dependent tasks, there is evidence linking adipose RBP4

in body weight regulation, insulin resistance, and glucose metabolism (Kotnik, P. et al. 2011). The functionality of RBP4 in adipose tissue suggests that RBP4 in ghrelin cells may also contribute to these metabolic mechanisms, particularly since the expression level of RBP4 in ghrelin cells is comparable to that of white adipose tissue (Walker, A.K. et al. 2013).

Notably, the discovery of RBP4 secretion implies that ghrelin cells may contribute to an alternative downstream signaling pathway (other than ghrelin pathways) to regulate insulin resistance, obesity, and diabetes. In particular, RBP4 has been recognized for its potential causative role in insulin resistance. Elevations in RBP4 via pharmacologic administration or genetic overexpression induce insulin resistance in mice (Yang, Q. et al. 2005). Serum RBP4 levels are significantly elevated in multiple mouse models of insulin resistance and obese or diabetic insulin-resistant humans (Yang, Q. et al. 2005; Graham, T.E. et al. 2006). Meanwhile, genetic deletion of RBP4 in mice increases insulin sensitivity, pointing out RBP4 as a potential new target for treatment of insulin resistance (Yang, Q. et al. 2005). Investigating the mechanism behind RBP4-mediated insulin sensitivity will prove useful as manipulations of molecular players in this pathway may have therapeutic efficacy for patients with diabetes. If RBP4 synthesized by ghrelin cells plays a systemic role and partakes in the development of insulin resistance, blocking ghrelin cell secretion of RBP4 may be beneficial in treating type-2 diabetes. While inhibiting RBP4 secretion in ghrelin cells might lower overall circulating ghrelin, it would not completely abolish circulating RBP4 since the liver secretes a large amount of RBP4. Hence, RBP4 from the liver could still carry out necessary retinol-related functions, but the reduced total levels of circulating RBP4 would potentially improve insulin sensitivity. However, it remains unknown whether RBP4 works in conjunction with TTR to have its effects on insulin sensitivity; this function may or may not be retinol-dependent. Solely changing RBP4 plasma levels may not be sufficient to induce deviations in insulin function. Thus, it is essential to consider TTR's role in RBP4-related functions. Variations in TTR levels can also largely

mediate levels of plasma RBP4 by controlling the degree of RBP4 glomerular filtration in the kidneys. Patients with type-1 diabetes displayed reduced RBP4 levels, which was partly attributed to loss during glomerular filtration as a result of low TTR levels (Pullakhandam, R. et al. 2012). Meanwhile, elevated TTR levels prevented RBP4 filtration in type-2 diabetes patients, contributing to an accumulation of circulating RBP4 (Pullakhandam, R. et al. 2012). Therefore, manipulations in TTR can also affect RBP4 levels.

Interestingly, RBP4 levels and ghrelin levels seem to have an inverse relationship in certain metabolic states. For example, obesity and type-2 diabetes are associated with reduced plasma ghrelin but elevated RBP4 concentrations (Poykko, S.M. et al. 2003; Yang, Q. et al. 2005; Graham, T.E. et al. 2006; Barazzoni, R. et al. 2007). Weight loss achieved by diet, exercise, or bariatric surgery decreases RBP4 levels, but elevates ghrelin levels (Cummings, D.E. et al. 2002; Weigle, D.S. et al. 2003; Reinehr, T. et al. 2005; Graham, T.E. et al. 2006; Janke, J. et al. 2006; Haider, D.G. et al. 2007; Santosa, S. et al. 2007; Vitkova, M. et al. 2007; Lee, J.W. et al. 2008). TTR levels in the stomach are also reduced after caloric restriction (Walker, A.K. et al. 2013). As a side note, although my study did not reveal differences in whole stomach RBP4 expression after caloric restriction, ghrelin cells only constitute a small portion of the whole stomach cell population. These inverse relationships are of particular interest since they indicate that RBP4 and ghrelin may act in opposition to one another, providing ghrelin cells with the tools to regulate metabolism in both directions. Further supporting the hypothesis of divergent RBP4 and ghrelin regulation, in this study RBP4 secretion was not stimulated by manipulations that lead to robust ghrelin secretion (caloric restriction, catecholaminergic or octanoic acid application). These clues provide useful information as to how follow-up studies should be conducted, mimicking conditions in which ghrelin secretion is low or blocked in order to potentially enhance RBP4 secretion.

As mentioned in chapter four, the sites of RBP4 and TTR biosynthesis may influence

their particular function, proposing tissue-specific roles for these proteins rather than a single, general task. Therefore, revealing the high expression of RBP4 and TTR in ghrelin cells opens the door of possibilities in ascertaining potential new stomach and pancreatic-specific roles for these proteins. This principle of a localized function is probable considering the proportion of RBP4 secreted from ghrelin cells is minimal compared to that secreted from the liver. Hence, the systemic effect of circulating RBP4 from ghrelin cells may not be apparent amidst the mass of RBP4 secreted from the liver. The observation that RBP4 secretion occurs in pancreatic ghrelin cells is particularly intriguing since pancreatic β-cells secrete insulin. Localized RBP4 and TTR activity in the pancreas may somehow modify insulin to diminish its functional capability, leading to insulin resistance. Similarly, RBP4 and/or TTR may act on β-cells to affect levels of insulin secretion. Alternatively, TTR may serve some functional importance in the pancreas or stomach, and RBP4 may act by keeping a pool of this hormone near the ghrelin cell. Further studies must unearth new findings in order to truly understand how RBP4 and TTR operate in and around the ghrelin cell.

Chapter Four Future Directions

Ghrelin cell secretion of RBP4 should definitely be explored further using contrasting conditions to those which stimulate ghrelin secretion. Since activation of beta-1-adrenergic receptors in ghrelin cells is known to strongly increase ghrelin secretion, it is possible that blocking these receptors will stimulate RBP4 secretion (Engelstoft, M.S. et al. 2013; Mani, B.K. et al. 2014). Blocking other receptors that stimulate ghrelin secretion such as the secretin receptors, melanocortin 4 receptor, or the composite receptor for the sensory neuropeptide CGRP may also influence RBP4 secretion in ghrelin cells (Engelstoft, M.S. et al. 2013). Oppositely, stimulating receptors that inhibit ghrelin secretion may also increase RBP4 release,

such as the lactate receptor, GPR81, or somatostatin receptors SSTR1, SSTR2, and SSTR3 (Engelstoft, M.S. et al. 2013).

Investigating the effects of obesity and diet on RBP4 expression in the gastric mucosa can also be studied by manipulating the diet and body composition of mice. RBP4 mRNA levels may be elevated in the gastric mucosa of mice placed on a long term high fat diet, and ghrelin cell RBP4 secretion may also increase with the induced obesity. Elaborating upon these studies, diet induced weight loss could be simulated by placing obese mice on a low calorie diet, and the RBP4 levels in the gastric mucosa could again be monitored. These studies could also be implemented in the ghrelin-hrGFP mice, followed by FAC-sorting and specific analysis of RBP4 in ghrelin cell-enriched pools. Likewise, it would also be wise to replicate the caloric restriction experiment performed in chapter four using ghrelin-hrGFP mice rather than C57BL/6J, allowing the separation of ghrelin cells via FAC-sorting.

In order to pursue the hypothesis that RBP4 may be acting locally in the stomach, studies can be carried out to establish whether the RBP4 receptor, STRA6, or retinoic acid receptors are expressed in different regions/cell types of the stomach (Muenzner, M. et al. 2013). Actually, retinoic acid, a metabolite of retinol, has been shown to fuel proliferation and differentiation of gastric epithelial progenitors and is associated with gastric cancer (Karam, S.M. et al. 2005; Kropotova, E.S. et al. 2013). Retinol is also involved in the gastrointestinal immune response and T-lymphocyte trafficking to the gastric mucosa (Kaufman, D.R. et al. 2011). Hence, the RBP4, TTR, and retinol complex may serve an essential purpose in gastric cells, and the local production of RBP4 and TTR in ghrelin cells may aide these specific actions.

The expression of STRA6 can also be measured in ghrelin-cell enriched pools or ghrelinoma cell lines to test for possible ghrelin cell expression, implying autocrine signaling. Immunohistochemical studies that double label STRA6 with different markers of gastric cell

populations may divulge where RBP4 is acting in the gastric community. These data would provide evidence for either autocrine or paracrine RBP4 signaling and accentuate the hypothesis of tissue specific RBP4 function.

In the end, the data in chapter four serve as great starting points in truly understanding the complexity of the ghrelin cell and its responsibilities in the network of gastric cells, as well as in the endocrine system as a whole. Taking a progressive step, this study focuses on metabolic proteins other than ghrelin in transcription, expression profiles, and secretion of the ghrelin cell. These projected future studies are the next step in constructing how these proteins, RBP4 and TTR, truly influence systemic metabolic regulation as well as local actions in the stomach.

Chapter Five Conclusions: *Characterization of gastric and neuronal populations using a transgenic mouse model*

In chapter five, my study focuses on the validation of the HDC-Cre mouse model designed in our lab and the characterization of these Cre-expressing cell populations. As a whole, the majority of histaminergic neurons and gastric cells contained Cre-recombinase activity, although regions E4 and E5 of the TMN do not share the high colocalization observed in other regions. Whereas the percent colocalization of Cre-recombinase activity and HDC expression in regions E1-E3 in the TMN ranges from 80.8% to 82.1%, regions E4 and E5 show 54.9% and 43.2% colocalization, respectively. Still, the specificity of Cre-recombinase activity to HDC-expressing cells was quite high since it was rarely observed in non-histaminergic cells. In fact, in the gastric mucosa and every region of the TMN, the percentage of cells with Cre-activity that co-expressed HDC was above 90%, reaching a pinnacle of 97.5% in region E2 of the TMN. Two brain regions in which Cre-recombinase activity was high even in the absence of HDC expression were the dorsal lateral geniculate nucleus and posterior thalamic nuclei.

As for the gastric population, FAC-sorting a highly purified population of Cre-recombinase expressing cells from the stomach mucosa allowed me to isolate cells with enhanced expression of HDC. Additionally, the increased expression of other ECL cell and mast cell markers in the Cre-recombinase cell population complemented the elevations in HDC. Immunohistochemical staining exhibited colocalization of HDC-expression and Cre-recombinase activity; toluidine blue was also detected in cells with Cre-recombinase acitivity. The hallmark morphological features of the elongated ECL cell and fried-egg shaped mast cell allowed me easily to identify these cell types. Through these methods, I was able to characterize specific Cre-recombinase activity in histaminergic ECL cells and mast cells.

Surprisingly, expression of gastrin was also highly enriched in the gastric cell population with Cre-recombinase activity. Further pursuit of this observation revealed a small percentage of HDC-immunoreactive cells also contains gastrin-immunoreactivity. The implications for this unexpected discovery are discussed in the section below (Chapter Five Significance).

Chapter Five Significance

After carrying out extensive work to validate the HDC-Cre mouse model, it can now be used in future experiments with certainty that the bulk of histaminergic cells will be targeted. Direct manipulation of the histaminergic system allows the study of multiple processes including histamine's mediation of gastric acid secretion, the immune response, circadian activities such as feeding behavior, sleep regulation, stress, mood, learning, and memory (Haas, H.L. et al. 2008).

In relation to ghrelin, brain histamine has been consistently shown to play a role in food intake and energy metabolism (Ookuma, K. et al. 1989; Kurose, Y. et al. 1999; Endou, M. et al.

2001; Masaki, T. et al. 2003; Gotoh, K. et al. 2013). A recent study has exhibited an interaction between nesfatin-1 and histamine signaling as they pertain to feeding behavior (Gotoh, K. et al. 2013). It was observed that the anorectic effect of nesfatin-1 was attenuated in histamine H1 receptor knockout mice. This connection is relevant because of the recent discovery of nesfatin expression and secretion in ghrelin cells (Stengel, A. et al. 2010; Stengel, A. et al. 2013), which links ghrelin cell endocrine signaling with neuronal histamine signaling. More directly, ghrelin has been found to affect TMN neurons in culture, as its application was discovered to inhibit G protein-coupled inward rectifier K+ channels (Bajic, D. et al. 2004)

In the stomach, the high colocalization of Cre-recombinase activity in ECL cells has valuable implications for future studies involving gastric acid secretion. Histamine plays a major role in stimulating HCl acid secretion from parietal cells, so ascertaining every aspect of ECL cell regulation is crucial for the management of acid-peptic disorders. Various disease states have increased gastric acid secretion such as Zollinger-Ellison syndrome (ZES) or duodenal ulcer disease, while other diseases such as pernicious anemia or atrophic gastritis present low or absent acid secretion (Chu, S. et al. 2012). The HDC-cre mouse model allows for specific manipulation of ECL cells which may provide clues as to the etiology of these diseases. In other words, breeding HDC-cre mice with mice containing a gene flanked by loxP sites will result in ECL cell-specific deletion of gene expression. Blocking ECL cell expression of a particular gene may result in disrupted gastric acid secretion, providing mechanistic pathways for ECL cell involvement in the development of certain gastric diseases.

Furthermore, ECL cell culture studies have uncovered numerous agents that affect histamine secretion, but only a few ECL cell receptors have been identified so far (Prinz, C. et al. 1993; Sandor, A. et al. 1996; Lindstrom, E. et al. 1997; Sachs, G. et al. 1997; Hakanson, R. et al. 2001; Lindstrom, E. et al. 2001; Oh, D.S. et al. 2005). Therefore, there are likely undiscovered receptors in ECL cells that play an important role in histamine secretion.

Performing a gene chip screen with mRNA obtained from FAC-sorted Tmt-enriched cells from HDC/Tmt mouse stomachs would aid in characterizing a transcriptional profile for ECL cells. Moreover, it was discovered that parathyroid hormone-like hormone (PTHLH) is expressed in ECL cells, but the function of this hormone as it pertains to the ECL cell remains unknown (Andersson, N. et al. 2005; Liu, C. et al. 2011). Serum gastrin levels were found to positively correlate with *Pthlh* mRNA expression in the gastric mucosa, suggesting a possible regulatory mechanism through gastrin activation of CCK_2 receptors on ECL cells (Liu, C. et al. 2011). Future studies with the HDC/Tmt mouse model may enlighten the field as to novel ECL cell signaling pathways, secretory hormones, gene expression, and mechanisms that control ECL cell function.

The unexpected finding that HDC is detected in a small portion of gastrin expressing cells is noteworthy, particularly since contradictory results from previous studies have left this possibility an open topic of discussion. The morphology and localization of these cells to the gastric antrum suggests that they are G-cells expressing HDC rather than ECL cells expressing gastrin. A prior study using immunohistochemical techniques in rat tissue found similar results, but a disputing study claimed that the HDC-immunoreactivity in the antrum was a cross reaction with non-HDC proteins (Hunyady, B. et al. 1998; Zhao, C.M. et al. 2004). If this was truly the case, it is curious that my FAC-sorted HDC-enriched cell population would have such extreme elevations in gastrin expression. Advancing past the limitations of immunohistochemical techniques performed in the previous studies, my method of FAC-sorted isolation of HDC-expressing cells adds another supporting layer to the notion that some G-cells express HDC. The FAC-sorting technique highlights how the HDC-Cre mouse model may be used in combination with advanced techniques to study specific cell populatons. Of note, while it is possible that the enrichment of gastrin in the HDC-enriched population may be an artifact of non-specific Cre expression in G-cells as well as histaminergic cells, only extended studies can

elucidate these findings.

My evaluation of the HDC-Cre mouse model shed light on the uneven percentage of Cre-recombinase activity and HDC-expression across histaminergic regions in the TMN. This knowledge may be substantial when using this mouse model for future studies since the different TMN regions are suspected of being functionally diverse rather than homogenous. Importantly, when using the HDC-Cre model in a breeding scheme with another transgenic mouse to modify gene expression, a smaller portion of cells in TMN regions E4 and E5 will be affected.

Chapter Five Future Directions

With the homogeneity of histaminergic neural populations under question, studies focusing on the diverging roles and functional specificity of TMN regions E1-E5 will become increasingly meaningful. The ability to visualize distinct histaminergic neurons in HDC/Tmt mice permits electrophysiological studies in slice preparations and allows the selective capability of choosing a particular TMN region to study. It would be fascinating to examine potential differences in the electrophysiological properties of histaminergic neurons located in different TMN regions. This could be accomplished by placing multiple HDC/Tmt mouse brain slices (comprising the entire TMN) under identical conditions and recording the responses of neurons in TMN regions E1-E5 to different neurochemical and electrical stimuli. Application of ghrelin to the slices would also unveil whether ghrelin activity in the local network can mediate histaminergic neuron currents as was shown in TMN cell culture (Bajic, D. et al. 2004). As a side, researchers studying visual information processing can make use of the ectopic Cre-recombinase expression in the dorsal lateral geniculate nucleus, as this brain region is the part of the relay center for visual information received from the retina (Cudeiro, J. et al. 2006).

The HDC-Cre model will also facilitate histaminergic cell culture work, granting the ability to FAC-sort HDC-expressing cells to sequester a pure population. TMN cell culture studies have been performed previously, but the population of cells in the culture were heterogenous with more than one cell type rather than simply histaminergic neurons (Bajic, D. et al. 2004). In highly purified, FAC-sorted cells, the direct effect of ghrelin on histaminergic neurons can be pursued further. Perhaps, the effect of ghrelin on histaminergic neurons observed in earlier studies was an artifact of not having pure histaminergic cell populations (Bajic, D. et al. 2004).

The accurate reflection of histaminergic cells in the stomach by the HDC/Tmt mouse also makes studies of ECL cells in the gastric community more practical. The clarity and ease with which ECL cells can be identified in this reporter mouse opens up countless possibilities when studying morphology, distribution, function, and gene expression in response to different stimuli. Another stomach cell type, the gastric mast cell, can also be examined when testing the stomach's immune response. Breeding HDC/Tmt mice with ghrelin-hrGFP reporter mice would allow for clear visualization of both ghrelin cells and histaminergic cells. Stomach tissue from these mice could be immunohistochemically labeled for other markers of other enteroendocrine cell types such as somatostatin or gastrin. Furthering this idea, these cell populations could be visualized after various manipulations such as caloric restriction, CSDS, HFD-induced obesity, or gastric bypass surgery. Careful attention to morphological features, cell numbers, and localization of the different cell populations in each of these situations would provide a comprehensive picture of different enteroendocrine cell responses within the gastric community. Encouraging the specific study of histaminergic cells in the stomach will advance potential therapeutic treatments for problems such as irritable bowel syndrome, gastric reflux, peptic ulcer formation, and gastric carcinomas.

Overall Significance

The main goal of much of my research is to better understand the etiology and mechanisms of psychiatric disorders and addiction (such as food addiction) in hopes of developing better therapeutic treatments for these disorders. Many treatments have undesired side effects due the targeting of a general pathway that is involved in multiple processes. For example, although ghrelin is antidepressant, it also can result in weight gain and overeating due to its orexigenic properties. Thus, by exploring downstream mechanisms through which ghrelin has specific effects, it may be possible to set apart the positive antidepressant effects of ghrelin from the unwanted hyperphagic effects. In determining that ghrelin's proneurogenic effects contribute to its antidepressant characteristics, researchers can now target the promotion or protection of neurogenesis to elevate mood through compounds such as P7C3. By revealing that P7C3-related compounds protects against stress-induced depression, I have introduced a specific new method – the enhancement of neurogenesis – that may not result in aversive side effects with other medications. While my studies of P7C3-related compounds have only scratched the surface of its potential effectiveness for depression, it is definitely a strong starting point. Future studies will reveal whether this compound only protects against depression induced by stress and whether it is effective when given after the onset of depression rather than as a preventative measure.

I would also like to emphasize that studying the relationship between peripheral enteroendocrine cells and central signaling in the brain is crucial for understanding functional outputs- both behavioral and physiological. Peripheral gastric cells secrete signals that can act on the brain not only through bloodstream transportation and direct activation of receptors in the brain, but also through activation of the vagal nerve. The brain then processes these signals through a complex network of neural connections that ultimately results in a functional output. However, both parts of the system- the central nervous system and the periphery- are

necessary in regulating different homeostatic processes and behaviors. Disturbing the activity of peripheral enteroendocrine cells of the stomach can significantly disrupt hormone signals that may be important for the daily homeostatic processes of an individual, such as regulated feeding behavior. The activity of the brain is equally vital since the stimulation of neurons and sending of electrochemical messages are what control the final output. My thesis dissertation highlights the interaction between peripheral signals, such as ghrelin, and central nervous system operations, revealing the key point that many different systems of the body are functionally interconnected. Therefore, it is essential that every aspect of the enteroendocrine – brain axis is explored in depth in order to uncover the many pathways that regulate human behavior. My studies of ghrelin and its multimodal effects provide evidence for a strong link between metabolic disturbances and psychiatric disorders. Although I have investigated how the relationship between ghrelin and the brain modulates complex feeding behavior and mood regulation, there are many other peripheral hormones, pathways, and behaviors that remain unexplored.

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