GHRELIN: THE HUNGER HORMONE THAT ISN'T

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DEDICATION

To my parents. With this, I am one step closer to having a job.
Ten years down; seven-ish to go.

Ghrelin: The Hunger Hormone that Isn't

By

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DISSERTATION

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GHRELIN: THE HUNGER HORMONE THAT ISN'T

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Ghrelin is a 28-amino acid acylated peptide hormone secreted by endocrine cells in the stomach. It was first identified in 1999 and shortly thereafter shown to stimulate appetite when injected into rodents and humans. While ghrelin knockout mice have failed to show a decrease in appetite or bodyweight, the literature – as well as the lay press – continues to presume ghrelin levels are a mediator of appetite *in vivo.* For example, the suppression of ghrelin levels secondary to gastric bypass surgery is frequently invoked as a contributing factor in the resulting weight loss. In the literature, this incongruity has been rationalized as embryonic or neonatal compensation, a claim predicated on a study by Luquet et al. which showed that AgRP/NPY neurons (which express the ghrelin receptor and are thought to be the critical target for appetite stimulation) can be ablated without consequence in neonatal mice, while in adult mice ablation causes a rapid and profound loss of appetite. Widespread acceptance notwithstanding, the hypothesis that a reduction in ghrelin levels decreases appetite in adults has never been tested. We generated a mouse line expressing the simian diphtheria toxin receptor on ghrelin cells. With these mice we are able to rapidly ablate ghrelin cells in adulthood with the injection of diphtheria toxin.

Despite an 80-95% loss of circulating ghrelin, our mice show no decrease in appetite or body weight in the short or long term and become obese and hyperinsulinemic in response to high fat feeding. To investigate why ghrelin seems to be sufficient but not necessary for hunger, we injected increasing doses of ghrelin and measured both food intake and the resulting plasma concentration. We found that the threshold dose for an appetite response raised blood concentrations more than 50-fold above physiologic levels – well above the highest concentration we have observed even during extreme starvation. We show that at physiologic levels ghrelin is neither necessary nor sufficient for hunger and conclude that it is not a key regulator of appetite or weight gain in mice. Ghrelin's only essential role in mice appears to be the maintenance of plasma glucose during periods of starvation.

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PRIOR PUBLICATIONS

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2. **McFarlane, M. R.,** Liang, G., & Engelking, L. J. (2014). Insig proteins mediate feedback inhibition of cholesterol synthesis in the intestine. *The Journal of Biological Chemistry*, *289*(4), 2148–2156. doi:10.1074/jbc.M113.524041

3. Engelking, L. J., **McFarlane, M. R.**, Li, C. K., & Liang, G. (2012). Blockade of cholesterol absorption by ezetimibe reveals a complex homeostatic network in enterocytes. *Journal of Lipid Research*, *53*(7), 1359–1368. doi:10.1194/jlr.M027599

4. Peterson, K. A., Nishi, Y., Ma, W., Vedenko, A., Shokri, L., Zhang, X., **McFarlane, M. R.**, Barizabal, J.-M., Junker, J. P., van Oudenaarden, A., Mikkelsen, T., Bernstein, B. E., Bailey, T. L., Bulyk, M. L., Wong, W. H., & McMahon, A. P. (2012). Neural-specific Sox2 input and differential Gli-binding affinity provide context and positional information in Shh-directed neural patterning. *Genes & Development*, *26*(24), 2802–2816. doi:10.1101/gad.207142.112

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CHAPTER I: INTRODUCTION

The story of ghrelin began almost forty years ago with the identification of the Growth Hormone Releasing Peptides (GHRPs) by Cyril Bowers and colleagues (Bowers et al., 2006). The GHRPs represent one of the earliest examples of "reverse pharmacology," discovered even before the major physiological GH secretagogue: Growth Hormone Releasing Hormone (GHRH) (Guillemin et al., 1982; Rivier et al., 1982). After the latter was identified, biochemical studies established that the GHRPs and other molecules in their class, collectively termed "growth hormone secretagogues" did not act through the same signaling pathway as GHRH and must bind a unique receptor. It would be twenty years before the endogenous receptor responsible for GHRP-stimulated GH-release was cloned (Howard et al., 1996). Termed the Growth Hormone Secretagogue Receptor (GHSR) (for review see (Cruz and Smith, 2008)), the purification of its endogenous ligand revealed an interesting twist: unlike GHRH, which as a classic Guillemin/Schally releasing factor is secreted by the hypothalamus through the hypophyseal-portal system, the GHSR ligand was present not in the brain, but rather in the stomach: a 28-amino acid acylated peptide its discoverers – Kenji Kangawa's laboratory – named "ghrelin" (Kojima et al., 1999; Kojima and Kangawa, 2005). Ghrelin was found to be secreted by a hitherto cryptic cell type, the X/A cell, which comprises 20% of the endocrine population of the oxyntic mucosa (Date et al., 2000).

Most beguiling for a society stricken by an obesity epidemic, ghrelin's secretion by the viscera implicated it as a hormonal link from the digestive system to the hypothalamus. Perhaps the ghrelin axis was a critical regulator of energy balance, much like the newly identified leptin axis, which only five years earlier had linked a fat-secreted ligand (Halaas et al., 1995) to a hypothalamic receptor (Tartaglia et al., 1995) – both of which (ligand and receptor) had already

been shown to be central regulators of body weight in humans (Clément et al., 1998; Montague et al., 1997). Additionally, prior studies with GHRPs had suggested that activation of the GHSR stimulated food intake, making ghrelin a tantalizing target (Bowers et al., 2006).

Speculation about ghrelin's role in energy balance was given hasty confirmation in the work of Matthias Tschöp, who showed that repeated injections of high-dose ghrelin caused rats to gain weight (Tschöp et al., 2000). Soon, an explosion of papers catapulted ghrelin to the forefront of obesity research. In what became the central dogma of ghrelin function, further work from Kangawa's lab implicated ghrelin in the direct stimulation of the central feeding neurons of the hypothalamus – the AgRP/NPY neurons in the arcuate nucleus – and suggested both that ghrelin blockade could reduce appetite and that a functional antagonism existed between ghrelin and leptin (Nakazato et al., 2001). Tamás Horváth at Yale stretched this model further, claiming that ghrelin was expressed in the hypothalamus itself (Cowley et al., 2003). Meanwhile, Stephen Bloom's lab showed that ghrelin injection stimulates appetite in humans as potently as it does in rats (Wren et al., 2001), and other researchers – including Matthias Tschöp and David Cummings – showed that human plasma levels rise before meals and fall rapidly thereafter (Cummings et al., 2001; Tschöp et al., 2001a) (for recent measurements using more accurate techniques see (Liu et al., 2008)). Multiple groups showed that ghrelin levels were perturbed in human obesity (English et al., 2002; Tschöp et al., 2001b), and in Prader-Willi syndrome, a genetic disease with a strong hyperphagic phenotype (Cummings et al., 2002a). Moreover, further work by Cummings suggested that gastric bypass surgery, the most effective therapeutic means of suppressing appetite and curing obesity, dramatically reduced ghrelin secretion (Cummings et al., 2002b).

Amidst the flurry of high profile publications in *Nature*, *Neuron*, *JCI*, and the *New England Journal of Medicine* proclaiming ghrelin the central molecule in the most important epidemic in modern medicine, Roy Smith's lab published what was perhaps the most significant result in all of ghrelin research in the journal *Molecular and Cellular Biology*: the first mouse with a germline knockout of the ghrelin gene, which, belying ghrelin's confirmed centrality, had no obvious phenotype (Sun et al., 2003). Ghrelin, the authors concluded, "is not critically required for viability, fertility, growth, appetite, bone density, and fat deposition, and not likely to be a direct regulator of leptin and insulin." Shortly thereafter, Mark Sleeman's lab independently deleted the ghrelin gene and showed a similar result (Wortley et al., 2004). Smith's subsequent genetic knockout of the GHSR also showed no indication that ghrelin played a role in appetite or body weight regulation, but proved definitively that the GHSR was responsible for both the GH- and appetite-stimulatory effects of exogenous ghrelin (Sun et al., 2004).

The exuberance over peripheral ghrelin's central role in feeding turned to puzzlement over its genetic dispensability in all food-related matters. In an attempt to reconcile this disparity, two groups claimed small reductions in weight gain when mice without ghrelin (Wortley et al., 2005) or ghrelin receptors (Zigman, 2005) were fed a high-fat diet in the early post-weaning period, leading to speculation that ghrelin could play a role in the development of a food intake set-point and that mice born without ghrelin could simply compensate for its absence by relying on other signals (Grove, 2005). Compensation proved to be an enduring rationalization for the discrepancy between ghrelin's robust pharmacologic effects and the lack of a demonstrable phenotype when deleted genetically.

This reasoning was not mere speculation; the phenomenon of neonatal compensation in feeding circuits is well supported in the literature. Initial single and compound knockouts of the key orexigenic neuropeptides, AgRP and NPY, did not affect food intake (Erickson et al., 1996; Qian et al., 2002). However, when Richard Palmiter's laboratory ablated the NPY/AgRP-expressing neurons themselves using diphtheria toxin (DTX), an interesting result emerged: in neonates, ablation of these neurons had no effect, but adults experienced a dramatic loss of appetite (Luquet et al., 2005). Combined with a contemporary publication from Jeffrey Flier demonstrating that CNTF-induced neurogenesis in the hypothalamus could alter energy homeostasis, (Kokoeva et al., 2005) this provided powerful support to the notion that the hypothalamus could adapt to a germline knockout of ghrelin. Indeed, in the very same AgRP/NPY knockout model, Palmiter's lab went on to show that mice were insensitive to GHSR stimulation of food intake after neonatal AgRP/NPY neuron deletion ((Luquet et al., 2007) mirroring an earlier result in *Agrp*$^{-/-}$*; Npy*$^{-/-}$ double knockout mice (Chen, 2004)). If mice could adapt to the neonatal loss of ghrelin's target neurons and their effector peptides, surely they could adapt to the loss of ghrelin.

Authors committed to maintaining the relevance of ghrelin in bodyweight homeostasis continue to cite Palmiter's result in diminishing the importance of the negative germline knockout data (Briggs and Andrews, 2011), maintaining that to truly rule out a role for ghrelin in food intake (and hence as an obesity drug target) one would need the analogous experiment, an inducible ghrelin knockout mouse. This has been often proposed but heretofore never performed.

Two major advances led to a resurgence of interest in ghrelin in the latter part of the 2000s. The first was the identification of Ghrelin o-acyltransferase (GOAT) in our laboratory (Yang et al., 2008a), and elsewhere (Gutierrez et al., 2008). This enzyme is required for the

addition of the octanoyl moiety to Ser-3 of ghrelin, a post-translational modification that is necessary for interaction with the GHSR. Moreover, Yang went on to show that the activity of GOAT could be robustly inhibited by a ghrelin peptide mimetic (Yang et al., 2008b) – if compensation were truly masking an important role for ghrelin in regulating energy balance, GOAT would be an ideal anti-obesity drug target. The second advance emerged from studies by Roy Smith in which ghrelin and GHSR knockout mice were studied under both positive and negative energy balance (Sun et al., 2007). While abrogation of ghrelin signaling was not meaningful in the setting of overeating, both models demonstrated slightly lower blood glucose when restricted to 50% of their normal caloric intake despite similar weight loss. NB: A prior study by Smith also suggested that ghrelin was involved in glucose control: in the setting of the profound hyperphagia of leptin deficiency ($Lep^{Ob/Ob}$), ghrelin-deficient mice ($Lep^{Ob/Ob}$; $Ghrl^{-/-}$) were resistant to hyperglycemia but not weight gain when extremely high body weights were achieved (Sun et al., 2006).

The observation that ghrelin deficiency predisposed mice to starvation-induced hypoglycemia was built upon in our laboratory to uncover the essential function of ghrelin: the maintenance of life-sustaining levels blood glucose in the setting of severe starvation. By studying $Goat^{-/-}$ mice (which have no octanoylated ghrelin), Zhao revealed that in the setting of severe caloric restriction (40% of normal calories), starved ghrelin-deficient mice become profoundly hypoglycemic (Zhao et al., 2010a) when fat mass is depleted below 2%. In this state, $Goat^{-/-}$ mice have attenuated levels of GH, and GH is capable of rescuing the hypoglycemia, suggesting that ghrelin-induced stimulation of GH, alone or in combination with other actions of ghrelin, is essential to maintain blood glucose in periods of starvation. Later studies have built on

this observation to implicate impaired gluconeogenesis in the development of hypoglycemia (Li et al., 2012).

Additionally, the creation of a ghrelinoma cell line in our laboratory revealed catecholamine-induced activation of β1-adrenergic receptors as the signal for the release of ghrelin (Zhao et al., 2010b) and led to the development of a model of the ghrelin axis in which starvation-sensing adrenergic neurons stimulate ghrelin-secretion to protect against hypoglycemia (Goldstein et al., 2011).

In the meantime, work on ghrelin receptors in other parts of the brain have endeavored to implicate ghrelin signaling in a variety of neuropsychiatric processes including learning and memory (Diano et al., 2006) and the dopamine system (Abizaid et al., 2006; Andrews et al., 2009), asserting roles in mediating depression (Lutter et al., 2008) and food-reward behavior (Chuang et al., 2011; Perello et al., 2010). Others have investigated ghrelin in the context of nutrient sensing of dietary lipids (Kirchner et al., 2009) and have published studies on ghrelin resistance in obesity (Briggs et al., 2010; 2013).

Indeed, work on and involving ghrelin continues apace. A pubmed search of "ghrelin" returns an average of ~700 results, including ~100 reviews articles, **per year** from 2009-2014. Judging from their introductions, many studies were undertaken with the assumption that ghrelin is indeed grossly important for food intake and bodyweight homeostasis – that the model of ghrelin's central role in the hypothalamic regulation of food intake developed by Kangawa 13 years ago remains valid (Nakazato et al., 2001). This assumption takes for granted that the negative genetic knockout data is inadmissible evidence because of some form of embryonic or neonatal compensation. Meanwhile, medical school curricula, including that of UTSW, continue to include ghrelin signaling in models of energy regulation. High impact journals publish papers

predicated on this assumption (Barnett et al., 2010), and clinicians develop techniques to inhibit ghrelin in hopes that it will cause weight loss (Arepally et al., 2008; Bawudun et al., 2012; Paxton et al., 2013). But despite the proliferation of literature and propagation of the model, the hypothesis that ghrelin suppression in an adult could reduce food intake has never been directly tested.

Philosophically, the statement "ghrelin does not regulate appetite *in vivo*," is impossible to prove right. One can only fail to prove it incorrect. Indeed, if ghrelin is one small part in a highly redundant appetite stimulating system capable of immediate undetectable compensation, it may forever elude experimental detection. However, if that is the case, it is useless as an interventional target in human biology and is irrelevant for all practical purposes; minor phenotypes do not become therapies. Here, Richard Palmiter's work on the neonatal ablation is critical, as it established that an insult to the very neurons mediating ghrelin's stimulation of appetite could be inconsequential to neonates but devastating to adults, leaving open the possibility that an undetected but major phenotype may result from the ablation of ghrelin in adult mice. Certainly, if exogenous ghrelin stimulates appetite primarily through AgRP/NPY neurons, Palmiter's results not only support the idea that the normal feeding behavior of a germline ghrelin knockout could be explained by compensation, they *predict* that an anorexic phenotype, if it existed, would not be detectable by genetic deletion and could only be seen in an inducible model.

In order to probe the existence of compensatory mechanisms and resolve the longstanding paradox of ghrelin function, we ask whether inhibition of ghrelin signaling in an adult mouse affects food intake or body weight. To do so, we generated transgenic mice that express the diphtheria toxin receptor (DTR) specifically on ghrelin-secreting cells (designated

Ghrl-DTR mice). When injected with diphtheria toxin (DTX) in adulthood, Ghrl-DTR mice lost their ghrelin cells within 24 hr and experienced a decline in plasma ghrelin levels of 80-95%. Ghrelin levels were maintained below 80% of normal for at least four weeks and could be maintained at lower levels with repeated administrations of DTX. We found no change in food intake or body weight in the setting of ghrelin cell ablation in the short or long term.

CHAPTER II: RESULTS

Development and Characterization of the Ghrl-DTR Mouse

Knowing that inducible knockout models are highly variable in quality and understanding that only a rapid, robust, and enduring deletion of ghrelin would suffice to argue a negative result with any confidence, we initially pursued a diverse set of transgenic strategies to delete ghrelin in adulthood. A total of six different mouse lines comprising three separate methods of cell ablation were created and tested. Of these, only one, which we called Ghrl-DTR, produced a reduction in plasma ghrelin in response to administration of an inducing agent. The strategy is presented schematically in Figure 1. Ghrl-DTR mice were generated by crossing a Ghrelin-Cre mouse (kind gift of Dr. Jeff Zigman (Engelstoft et al., 2013)) to a flox-inactivated copy of the simian diphtheria toxin receptor (Buch et al., 2005). Expression of Cre recombinase removes a premature stop codon and activates transcription of *DTR*, conferring DTX sensitivity to ghrelin cells.

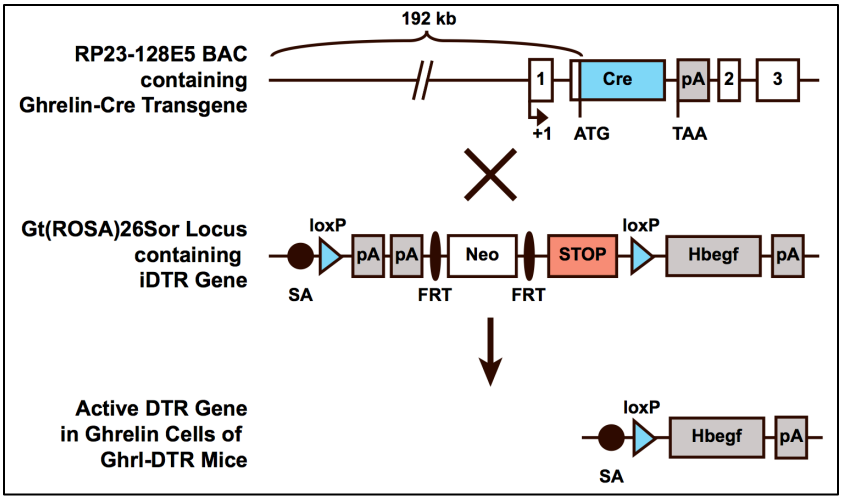

Figure 1. Schematic of Cross between Ghrl-Cre and iDTR Alleles to Generate Active DTR Molecules on Ghrelin Cells.

Diphtheria toxin, hereafter referred to as DTX, is a secretion of *C. diphtheriae* that abrogates protein synthesis by catalytic inactivation of Elongation Factor 2, killing the target cell. The system is both highly sensitive and highly specific. Sensitivity is conferred by the potency of the toxin: it has been shown that a single molecule of DTX is sufficient to kill a cell

(Yamaizumi et al., 1978). However, in order to enter cells, DTX must bind a protein called Heparin Binding EGF-like Growth Factor (*Hbegf* gene) (Naglich et al., 1992), hereafter referred to as the diphtheria toxin receptor (DTR). The mouse homolog of *Hbegf* is highly resistant to DTX, and therefore DTX-sensitivity in mice is limited only to cells that have been transgenically modified to express the simian (or human) DTR. This strategy has been used for many years to inducibly delete specific cell populations in transgenic mice (Saito et al., 2001), and was also the approach used by Luquet et al. to delete AgRP/NPY neurons in the study that was the primary motivation for our experiments (Luquet et al., 2005). In our Ghrl-DTR mice, a single IP dose of only 200 ng (8-10 ng/g BW) DTX dissolved in 200uL of saline was sufficient to robustly ablate ghrelin cells.

This system has the additional advantage of testing the notion that ghrelin cells may have other secretions that mediate appetite. Indeed, there is no dearth of proposed alternate secretions of ghrelin cells, including an alternate cleavage product of the ghrelin propeptide called "obestatin" (Zhang et al., 2005) as well as a hormone called nesfatin-1 and des-acyl ghrelin itself (Kerbel and Unniappan, 2012). While all of these results have all been strongly refuted, a secondary secretion is not out of the question, especially in light of the discovery of an alternate

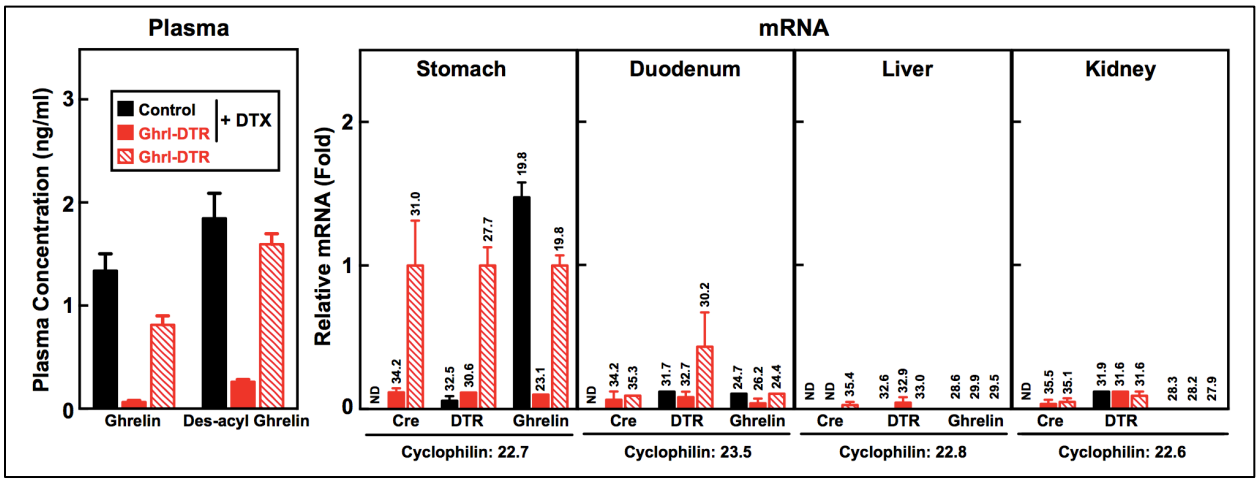

Figure 2. Plasma and mRNA Measurements from Control and Ghrl-DTR mice.

secretion of gastric inhibitor peptide (GIP)-producing K-cells called Xenin-25 (Wice et al., 2010).

As described in Experimental Procedures, mice with the genotype *Ghrl-Cre; iDTR$^{f/f}$* or *Ghrl-Cre; iDTR$^{f/+}$* were designated Ghrl-DTR, and littermates without the ghrelin-cre transgene (*iDTR$^{f/+}$* or *iDTR$^{f/f}$*) were used as control mice. As a first step towards characterizing the Ghrl-DTR mice, we performed a multiple tissue quantitative PCR assay to ensure that the flox-inactivated DTR gene was activated by the Ghrelin-Cre transgene specifically in stomach. In this study, chow-fed control and Ghrl-DTR (male, 8-week old) were injected with 8-10 ng/g body weight of DTX. Mice were sacrificed 3 days after injection. Blood was drawn from the inferior vena cava. Plasma ghrelin and des-acyl ghrelin levels were measured as described in Experimental Procedures. Stomach, duodenum, liver and kidney were harvested, mRNA was isolated from these tissues, and quantitative RT-PCR analysis of Cre, DTR, and ghrelin were performed on mRNAs from the indicated tissue. The number above each bar represents the mean C_t value from 3-4 mice. The results, shown in Figure 2 indicate highly specific recombination and activation of the DTR gene in stomachs of Ghrl-DTR mice. As expected, injection of DTX abrogated mRNA expression of Cre, DTR, and ghrelin, and caused a robust reduction of plasma ghrelin and des-acyl ghrelin. Prior to injection of DTX, ghrelin plasma and mRNA levels were alike in Control and Ghrl-DTR mice.

Next, we sought to establish a thorough time course of the DTX-mediated deletion using larger numbers of mice. In this study, chow-fed control and Ghrl-DTR littermates (male, 8-week-old) were injected intraperitoneally with 8-10 ng/g body weight of DTX at the indicated time prior to sacrifice. To obtain groups of n=6, two identical n=3 studies were performed on consecutive days and combined into one data set. Injections of DTX were staggered such that the

indicated time points were reached at 2 p.m. (the circadian maximum of ghrelin concentration) on the day of sacrifice. Uninjected controls are shown at time 0. After anesthetization by isoflurane, blood was drawn from the inferior vena cava, after which the whole stomach was divided in half longitudinally and immediately homogenized in RNA-STAT. Plasma measurements for ghrelin (A), des-acyl ghrelin (B), and insulin (C) were determined by ELISA as described in Experimental Procedures. mRNA levels for Ghrelin (D), chromogranin A (E), and TNFα (F) were measured by quantitative RT-PCR on stomach RNA and normalized to cyclophilin mRNA. Each value represents mean ± SEM of 6 mice, except the Ghrl-DTR groups at 8 hr and 3 days, which represent n=5 and n=4 mice, respectively. One mouse at the 8-hr time point and 2 mice at the 3-day time point did not respond to DTX as indicated by PCR of stomach RNA; the values for these mice were omitted from the data. **, $p < 0.01$; ***, $p < 0.001$.

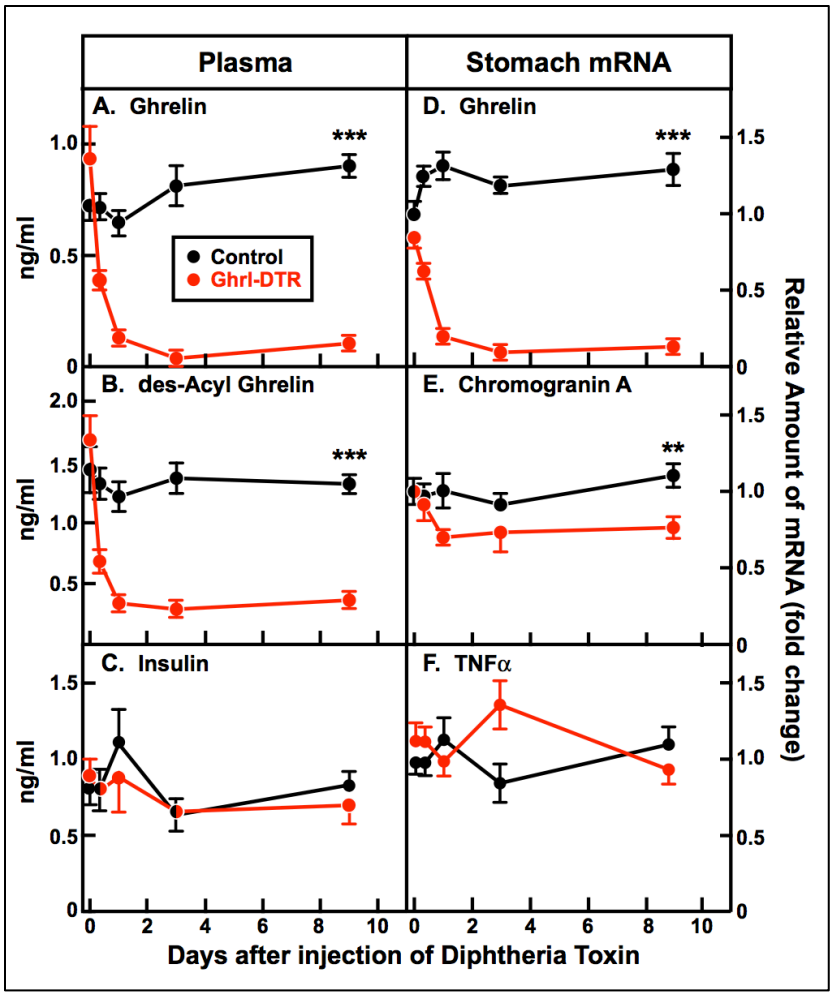

Figure 3. Time Course of Ghrelin Cell Deletion after DTX Injection

The results of this study, shown in Figure 3 indicate a rapid and robust response to DTX in Ghrl-DTR

mice; within only 8 hr of injection, Ghrl-DTR mice experienced a 50% reduction in plasma ghrelin. By 24 hr, the level had declined by 86%, and it remained at this low level through nine days (Figure 3A). The decline in des-acyl ghrelin was equally rapid, but not quite as profound, the level averaging 20% of normal through the 9 days (Figure 3B). Plasma insulin levels were unaffected by the DTX injection in either control or Ghrl-DTR mice (Figure 3C).

The level of ghrelin mRNA in stomach extracts declined in parallel with the fall in plasma ghrelin levels (Figure 3D). The mRNA encoding chromogranin A, a protein found in all gastric endocrine cells, fell by 29% in the Ghrl-DTR mice (Figure 3E). This is consistent with the estimate that ghrelin cells account for ~20% of chromogranin A-containing cells in the stomach (Date et al., 2000). We saw no significant increase in the mRNA encoding TNFα in the stomach extracts, which suggests that DTX did not elicit an inflammatory response in the stomach (Figure 3F).

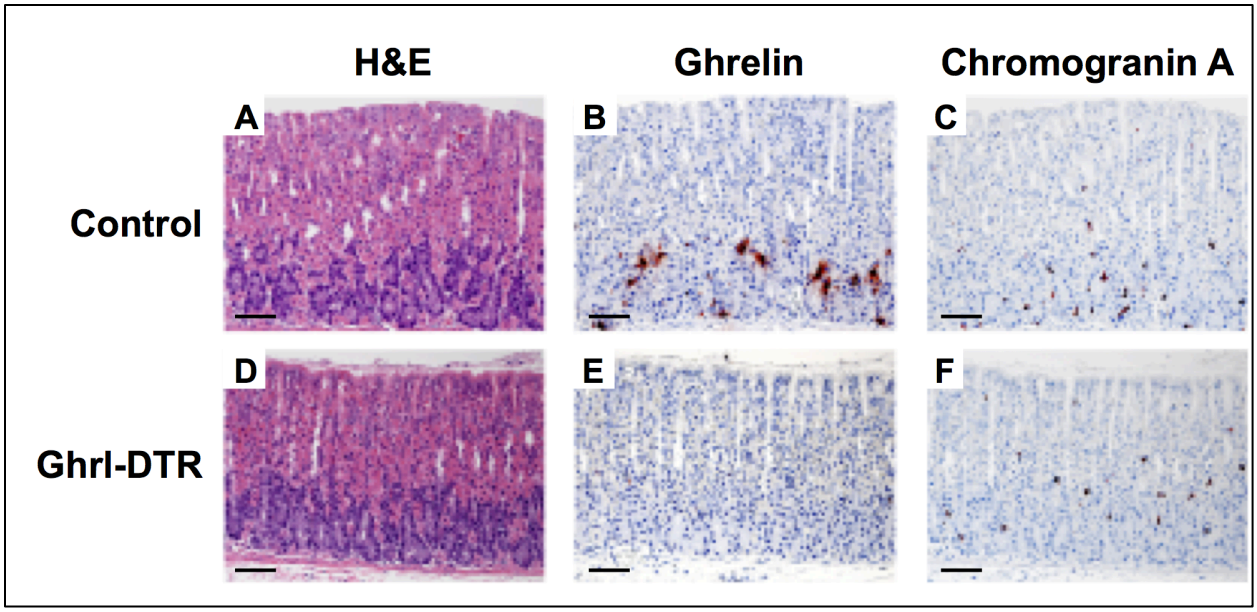

Figure 4. Histologic Sections of Gastric Fundus from Control and Ghrl-DTR Mice Magnification, 10x; scale bar, 250 µm.

To further verify the deletion of ghrelin cells in the stomach and ensure that the process did not damage the architecture of the stomach, we prepared histologic sections of stomachs of control and Ghrl-DTR mice that were killed 9 days after DTX injection. The animals were part of the group studied in Figure 3. Figures 4A and D show that stomach architecture was preserved in Ghrl-DTR mice after DTX injection. In control mice we easily detected cells that stained positive for ghrelin (Figure 4B). We saw no such cells in the stomachs from the Ghrl-DTR mice (Figure 4E). Abundant chromogranin A-positive cells were observed in control and Ghrl-DTR stomachs (Figures 4C and F). No histologic sign of inflammation was observed.

Ghrelin has been reported in cells in the fetal and adult pancreas (Date et al., 2002). While the investigators who characterized the Ghrelin-Cre mouse we used described an absence of reporter activation in islets (Engelstoft et al., 2013), and insulin secretion and blood glucose were unaltered after DTX injection (Figure 3C, 6, and 7) we sought to further verify that no histologic damage occurred after DTX injection. To do so, we prepared histologic sections of pancreases of control and Ghrl-DTR mice that were killed 9 days after DTX injection. The

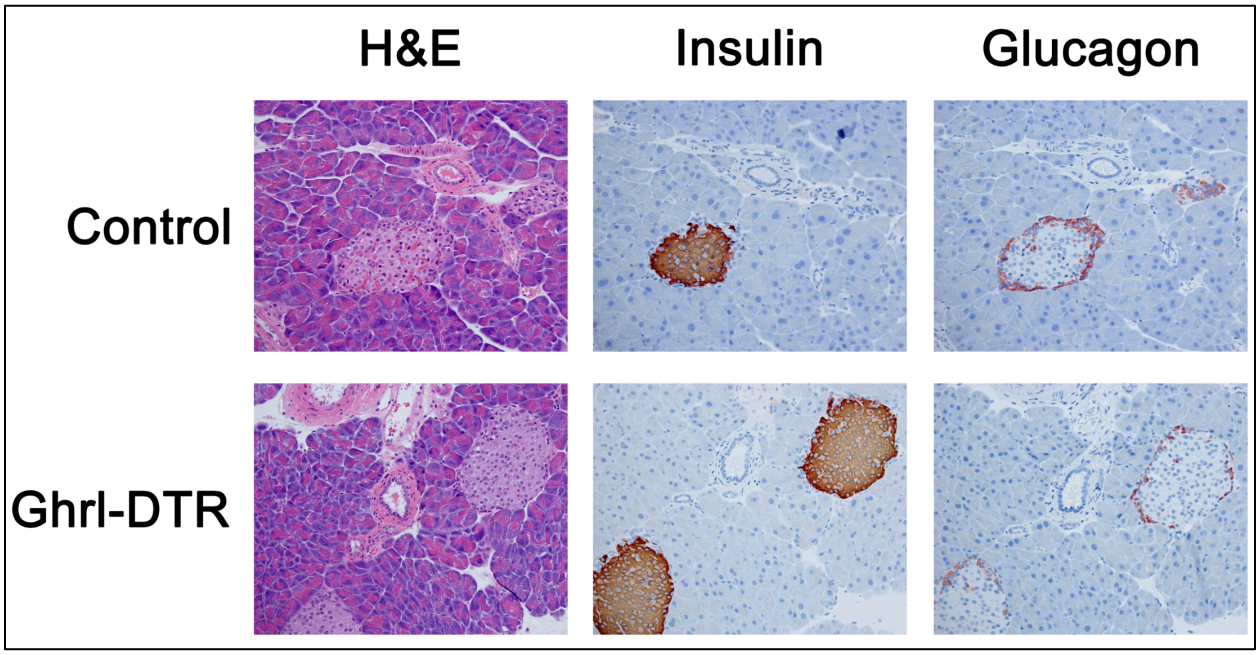

Figure 5. Histologic Sections of Pancreas after DTX Injection from Control and Ghrl-DTR Mice Magnification, 20x

animals were part of the group studied in Figure 3. Figures 5A and D show that pancreatic islet architecture was preserved in Ghrl-DTR mice after DTX injection. In both groups of mice we easily detected cells that stained positive for insulin and glucagon. No histologic sign of inflammation was observed. Staining with anti-ghrelin antiserum was negative on both control and Ghrl-DTR islets, indicating an absence of ghrelin in the adult mouse pancreas.

While some groups have indicated expression of ghrelin in hypothalamic neurons (Cowley et al., 2003), others have been unable to show such expression by mRNA, protein, or, most revealingly, X-gal staining in a mouse model where LacZ has been knocked into the ghrelin locus (Wortley et al., 2004). Additionally, characterization of the Ghrelin-Cre mouse also failed to show reporter activation in the brain (Engelstoft et al., 2013). We therefore did not perform experiments to rule out damage to the hypothalamus.

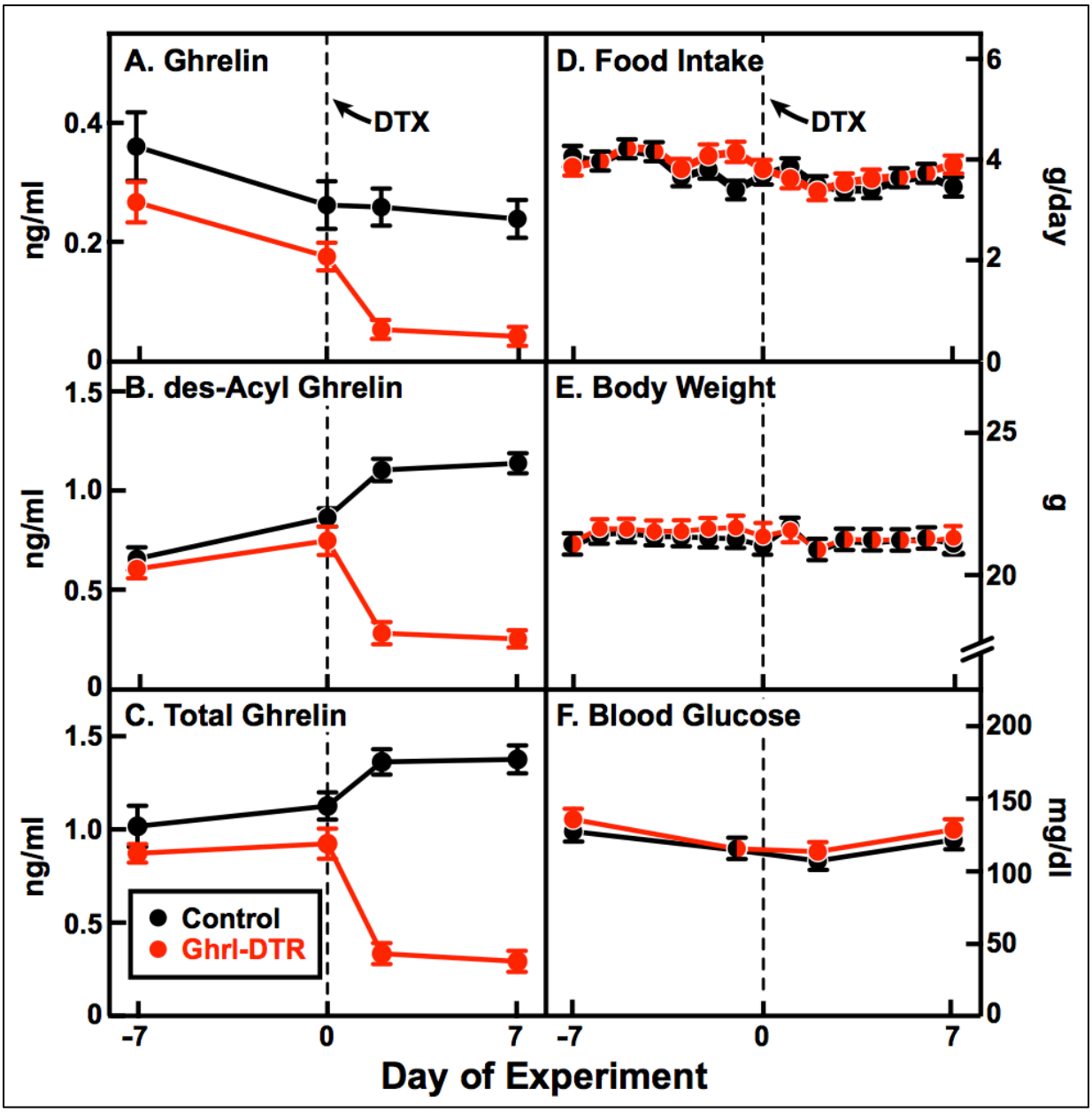

Figure 6. Body Weight and Food Intake after Acute Ghrelin Ablation

Observation of Body Weight and Food Intake after Acute Ghrelin Cell Ablation

Satisfied that the Ghrl-DTR mice provided a robust means of acutely ablating ghrelin cells in adult mice and that a 80%+ loss of circulating ghrelin was sufficient to make a strong conclusion about the function of ghrelin in normally developed adult mice, we proceeded to observe the feeding behavior of mice in the setting of acute ghrelin cell ablation. In Figure 6, chow-fed

control and Ghrl-DTR littermates (male, 8-week-old) were housed individually and injected intraperitoneally with DTX on day 0. Blood was drawn by tail vein at 2:00 p.m. on the indicated day. Ghrelin (A) and des-acyl ghrelin (B) levels were measured by ELISA, and added together to give total ghrelin (C). Food intake (D) and body weight (E) were measured daily at 2 p.m. Experimenters were blinded to the genotypes of the mice for body weight and food intake measurements. Blood glucose (F) was measured by Bayer Glucometer. Dotted lines denote time of DTX injection. Each value represents mean ± SEM of 8-9 mice. One mouse that did not respond to DTX (by ghrelin and des-acyl ghrelin measurements) was excluded.

On average, plasma ghrelin fell by 80% and des-acyl ghrelin levels by 76% in Ghrl-DTR mice relative to littermate controls. Despite these reductions in the 7 days following injection of DTX, we observed no alteration in food intake, which averaged 3.6 ± 0.1 g/day in both groups (Figure 6D) or body weight, which averaged 21.2 ± 0.3 g in both groups (Figure 6E). No differences in blood glucose levels were observed (Figure 6F).

Effect of Ghrelin Cell Ablation on Long Term Bodyweight and Food Intake Under Chow- and High Fat Diet-fed conditions.

Next, we sought to determine whether the loss of ghrelin affected food intake or weight gain over the long term and or, as others have reported (Wortley et al., 2005; Zigman, 2005), in response to high fat feeding. In this study, control and Ghrl-DTR littermates (male, 8-week-old) were housed in cages containing 4 mice of mixed genotype. Mice were fed either a chow diet or a HFD for 12 weeks as indicated and were injected intraperitoneally with DTX at the indicated time. Body weight (A) was measured weekly. Body fat (B) was measured monthly. Blood was drawn biweekly at 2:00 p.m. Plasma levels of insulin (C), ghrelin (D), and des-acyl ghrelin (E)

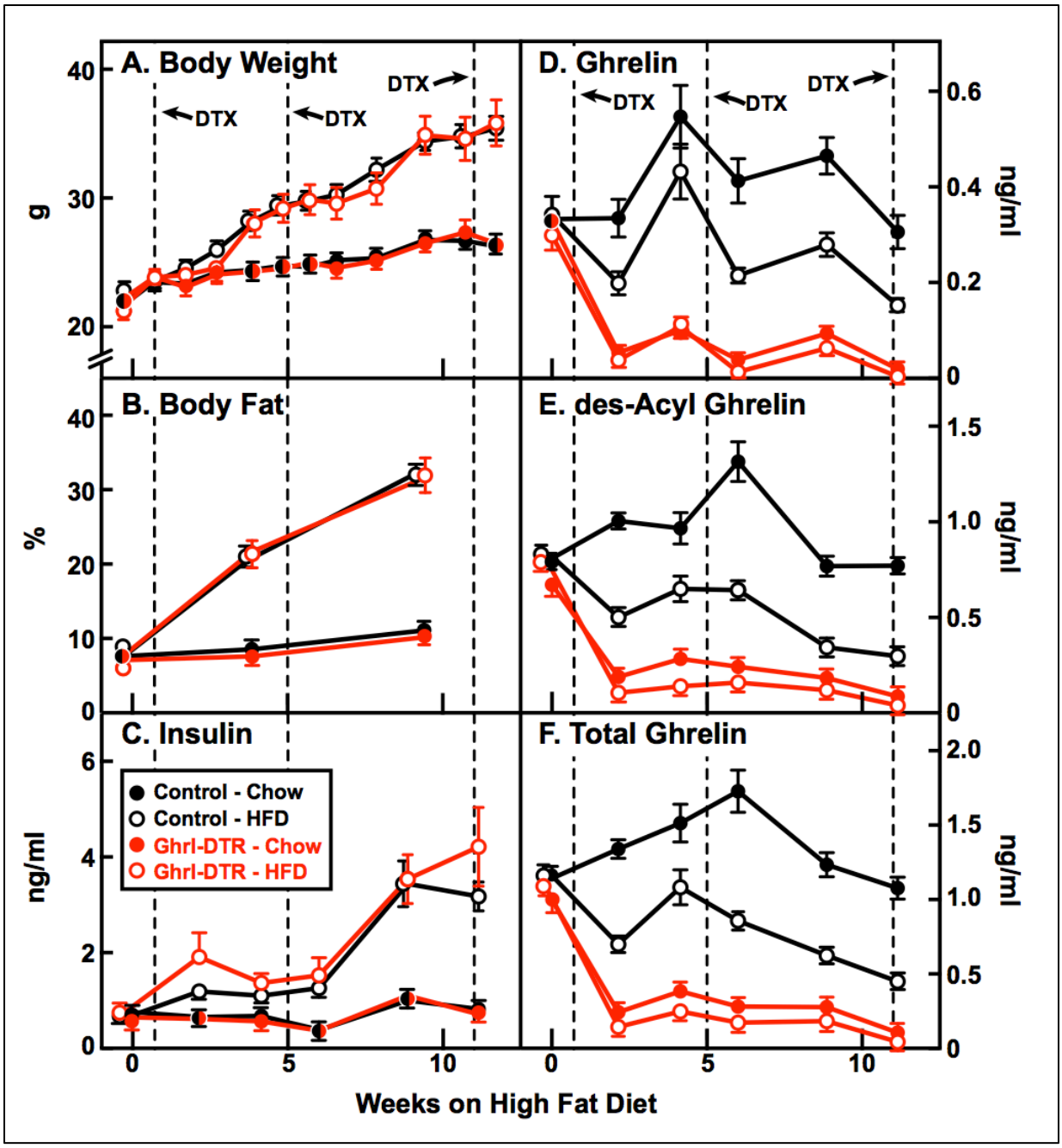

Figure 7. Long Term Effects of Ghrelin Cell Ablation under Chow- and High Fat Diet-Fed Conditions

were measured by ELISA, and values in (D) and (E) were added together to give values for total ghrelin (F). Dotted lines denote times of DTX injection. Each value represents mean±SEM of 11-15 mice. One control mouse was incorrectly genotyped, and three Ghrl-DTR mice in the chow-fed group did not respond to DTX; the values for these mice were omitted from the data.

HFD, high-fat diet. Investigators were blinded to genotype for all sample collections and assays.

In this study, control and Ghrl-DTR mice gained equal amounts of weight on both chow and the HFD (Figure 7A), and they exhibited equal increases in body fat (Figure 7B). Plasma measurements of insulin, ghrelin, des-acyl ghrelin, and total ghrelin were made biweekly at 2 p.m. in all mice (Figure 7C-F). Area under-the-curve calculations beginning after injection of DTX and extending until the end of the study indicate an average reduction in plasma ghrelin of 85% and 89% in chow- and HFD-fed Ghrl-DTR mice relative to chow-fed control mice (Figure 7D). Corresponding reductions in des-acyl ghrelin were 79% and 87%, respectively (Figure 7E). In control mice the HFD reduced plasma ghrelin by 38% and des-acyl ghrelin by 49% (Figures 7D and E). The significance of this finding is unknown, but is consistent with observations in humans showing that plasma ghrelin levels are significantly lower in obese subjects than in lean subjects (Shiiya et al., 2002). In the HFD-fed groups, hyperinsulinemia (> 3 ng/ml) developed in control and Ghrl-DTR mice between weeks 6 and 9 of high fat feeding (Figure 7C). These results convinced us that ghrelin cells are not important for controlling appetite and weight gain and that developmental compensation for loss of ghrelin does not occur.

Effect of Ghrelin Cell Ablation on Blood Glucose Control During Starvation.

We have shown previously that mice genetically deficient for acylated ghrelin (*Goat*$^{-/-}$) become severely hypoglycemic during 60% calorie restriction (Goldstein et al., 2011; Li et al., 2012; Zhao et al., 2010a). The same was true of genetic ghrelin deficiency (Li et al., 2012). To determine whether ablation of ghrelin cells in adulthood would reproduce this result, we subjected control and Ghrl-DTR mice (n=8) to seven days of 60% calorie restriction. These animals were fed each day at 6 p.m. Blood glucose was measured at 5:30 p.m. just before

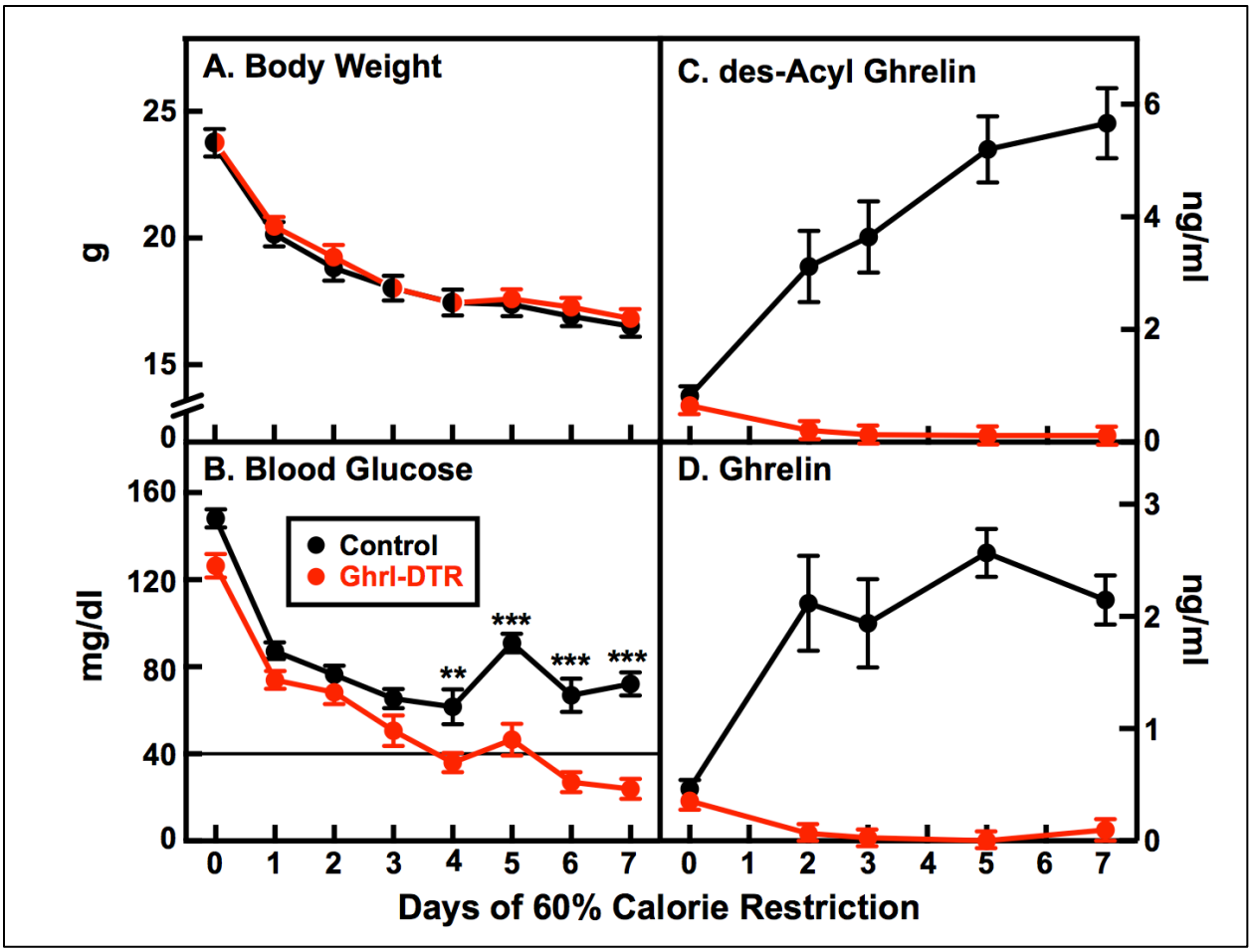

Figure 8. Effect of Ghrelin Cell Ablation on Blood Glucose During Calorie Restriction

feeding (Figure 8). Inasmuch as calorie-restricted wild-type and ghrelin-deficient mice consume all of their food within 1 hr (Zhao et al., 2010a), the mice in Figure 8 have been fasting for 23.5 hr when the blood is collected at 5:30 p.m. Beginning on day 4 and continuing through day 7 of calorie restriction, we observed a decline in blood sugar in Ghrl-DTR mice relative to control mice, with Ghrl-DTR mice reaching a nadir of 24 ± 4 mg/dL on day 7, while control mice averaged 72 ± 5 mg/dL (Figure 8B). These results are similar to those observed in calorie-restricted wild-type and *Goat*$^{-/-}$ mice (Zhao et al., 2010a), confirming that the presence of ghrelin during calorie restriction is necessary to protect against fasting-induced hypoglycemia. By day 7 of calorie restriction, ghrelin levels in control mice reached 2.2 ± 0.21 ng/ml, while levels in

Ghrl-DTR mice were undetectable (i.e. < 0.07 ng/ml) (Figure 8D). Des-acyl ghrelin levels reached 5.7 ± 0.62 ng/ml in control animals and were 0.12 ng/ml in Ghrl-DTR mice, a reduction of 98% (Figure 8C).

Food Intake and Plasma Concentration After Acute Ghrelin Injection

The failure of ghrelin cell ablation to reduce food intake in mice contrasts with many previous experiments that demonstrated that injections of ghrelin cause an acute increase in food consumption (Tschöp et al., 2000; Wren et al., 2001; Kojima and Kangawa, 2005). In an attempt to reconcile these findings, we injected doses of ghrelin ranging from 0.01 to 1 mg/kg to control and Ghrl-DTR mice). We performed the first round of these experiments comparing Control and Ghrl-DTR mice to verify that there were no differences in Ghrelin sensitivity as a result of the Ghrelin cell ablation. In the first experiment, chow-fed control and Ghrl-DTR littermates (male, 8-12 weeks old) were housed individually and injected with DTX on day 0. On day 2, plasma ghrelin and des-acyl ghrelin were measured to verify ablation in Ghrl-DTR mice (Figure 9) and

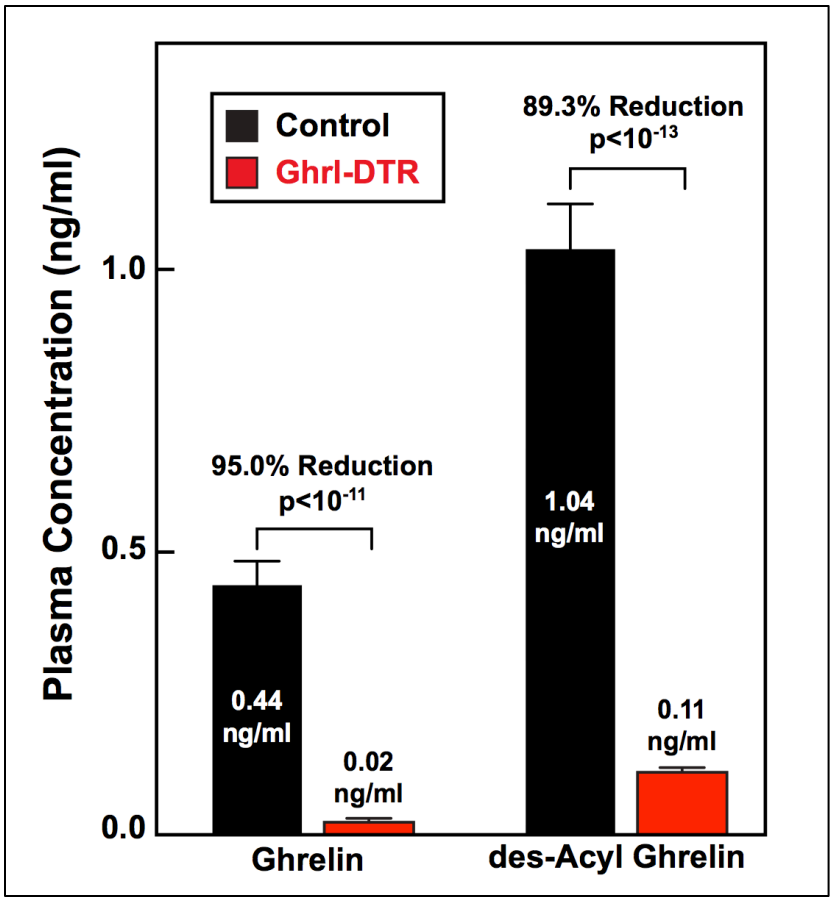

Figure 9. Verification of Ghrelin Deletion 2 Days after DTX Injection for Mice in Figure 10

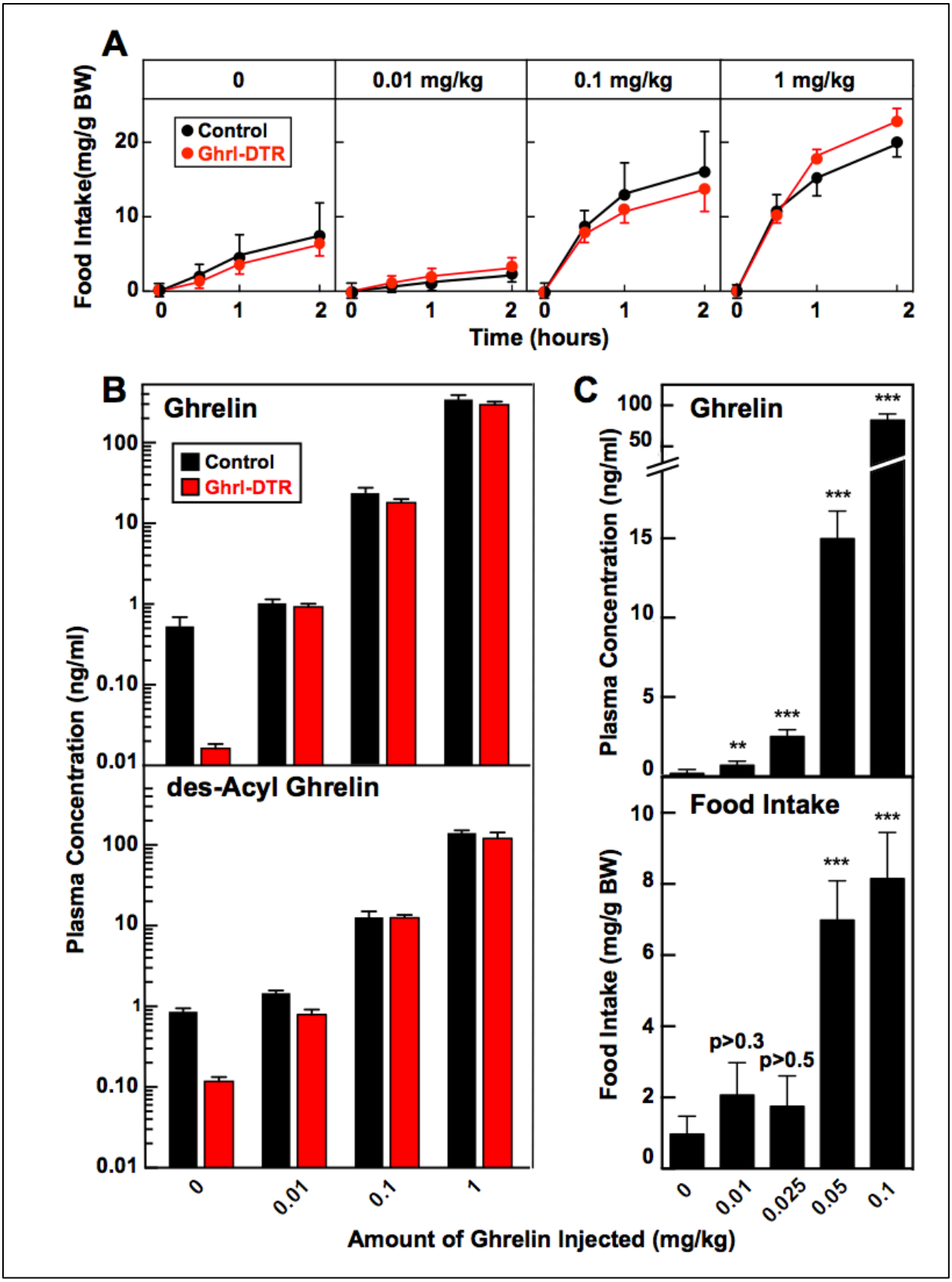

Figure 10. Plasma Concentration and Food Intake Following Acute Ghrelin Injection

mice were randomly sorted into 8 groups of 5 mice each. On day 4 at 2:00 p.m., mice of both genotypes were injected subcutaneously with 0.1 ml of 0.15 M NaCl containing the indicated amount of ghrelin. (Figure 10A) Food intake was measured at 0, 0.5, 1, and 2 hr after injection. (B) On the following day, an identical injection of ghrelin was given, and blood was drawn from the tail vein 10 min after injection of the indicated concentration of ghrelin. Levels of ghrelin (top) and des-acyl ghrelin (bottom) were measured by ELISA. (C) Chow-fed control and Ghrl-DTR littermates (not injected with DTX) were randomized into 5 groups of mixed genotype (n=6). On the first day of the experiment, mice were injected subcutaneously at 2:00 p.m. with the indicated dose of ghrelin (top), and food intake (bottom) was measured at 10 and 30 min, respectively, after injection as described in *A*. On the following day, identical injections of ghrelin were given, and blood was drawn from the tail vein 10 min later, as described in *B*. **, $p < 0.01$; ***, $p < 0.001$.

The experiments were initiated at 2:00 p.m. (5 hr after beginning of the light cycle), to minimize the baseline level of food intake – this is a time in which mice normally consume only small amounts of food. Injection of 0.01 mg/kg ghrelin failed to increase food intake in control and Ghrl-DTR mice (Figure 10A). At a 10-fold higher dose of ghrelin (0.1 mg/kg), there was a marked increase in food intake over the first 30 min in both control and Ghrl-DTR mice, after which there was little further increase. Another 10-fold dose increase (1 mg/kg) produced a slightly higher food intake at 30 min, and the mice continued to eat an increased amount over 2 hr. Again, the response of control and Ghrl-DTR mice was indistinguishable, indicating that loss of endogenous ghrelin does not increase sensitivity to the injected hormone.

We could not measure plasma ghrelin levels during the food intake experiments because it would have disrupted the feeding pattern of the mice. To obtain plasma for ghrelin

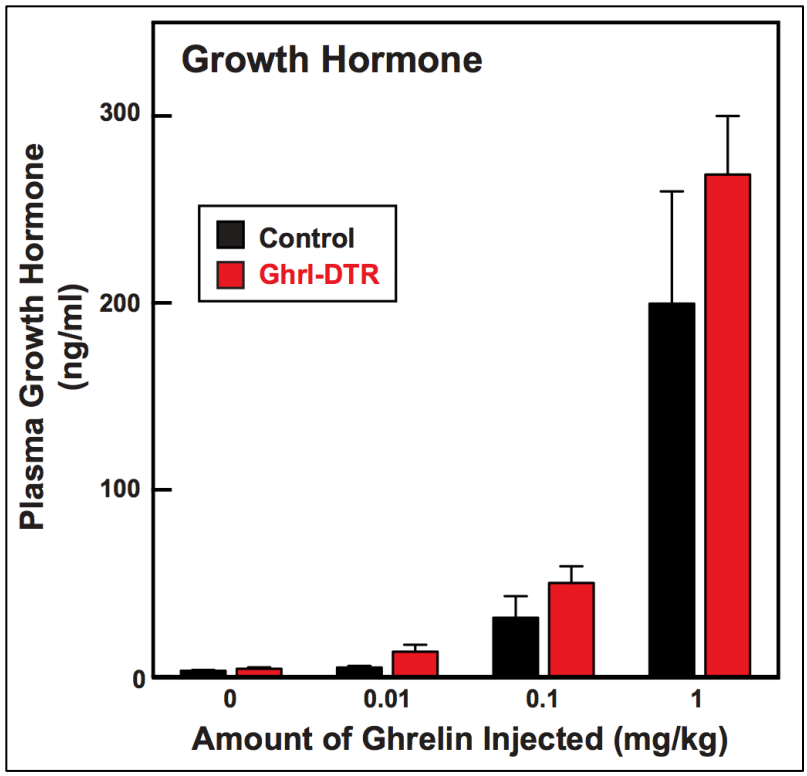

Figure 11. Dose-Response Curve of GH Release after Ghrelin Injection

determination, we repeated the saline or ghrelin injections on the next day at the same time and measured the plasma level of ghrelin and des-acyl ghrelin after 10 min. Plasma ghrelin was reduced by 97% in the saline-injected Ghrl-DTR mice as compared with controls. Injection of ghrelin at 0.01 mg/kg raised the plasma ghrelin level in the Ghrl-DTR mice so that it equaled the level in the control mice, yet there was no increase in food intake (Figures 10A and 10B). A dose of 0.1 mg/kg, which increased food intake, raised the plasma ghrelin level to 45 times above the value in saline-injected control mice. At 1 mg/kg, ghrelin injection raised the plasma ghrelin level to 650-fold above the saline-injected level (from 0.53 to 345 ng/ml) (Figure 10A, top). The values for des-acyl ghrelin paralleled those for ghrelin (Figure 10B, bottom). We measured plasma GH in the same samples and found that a ghrelin dose of 0.1 mg/kg was required to raise GH in control mice (Figure 11).

The experiment of Figures 10A and 10B indicated that in order to increase food intake acutely, plasma ghrelin must be elevated above physiological levels. To explore this phenomenon over a narrower concentration range, we repeated the ghrelin injections in control mice and measured food intake over the next 30 min (Figure 10C). On the next day, we repeated

the injections and measured plasma ghrelin 10 min later. Injection of 0.025 mg/kg of ghrelin raised the plasma level of ghrelin 14 times above the level in saline-injected mice, but it did not increase food intake significantly. When the dose was raised to 0.05 mg/kg, plasma ghrelin increased 80-fold, and food intake increased by 7.2-fold. A similar 8-fold increase in food intake was observed when the ghrelin dose was further increased to 0.1 mg/kg. Thus, in this experiment, as in the experiment of Figures 10A and 10B, it was necessary to raise plasma ghrelin many fold above normal in order to observe an acute increase in food intake.

CHAPTER III: DISCUSSION

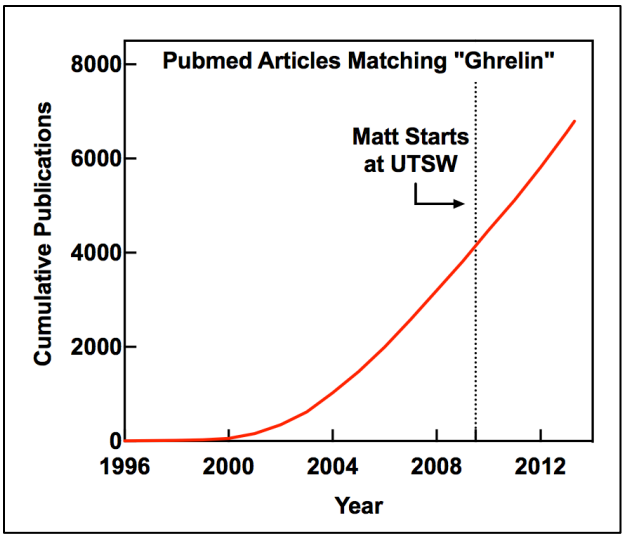

Figure 12. Cumulative Publications Matching the search term "Ghrelin" in Pubmed.

Determining the true physiologic role of ghrelin is an important problem. Since its discovery 15 years ago, almost 8000 papers have been published on or involving the hormone. The pace shows no signs of declining, with an average of 700 papers and 100 review articles published each year – a total of over 3000 publications since I began my first year of medical school in 2009 (Figure 12), where I learned that ghrelin acted as an important physiologic regulator of appetite, counteracting leptin in the arcuate nucleus of the hypothalamus. Indeed, the frenetic pace of work on ghrelin (and the funding available for such projects) can only be because of its reputation as a "hunger hormone." We live in overfed times, and understanding the physiologic regulation of food intake in a manner that could lead to an effective treatment for obesity remains a highly sought after goal in biomedical research. The relevance of all research undertaken on ghrelin towards these ends is predicated on a physiologic role in hunger stimulation. If ghrelin has, in fact, nothing at all to do with appetite – if its hunger stimulating effect is merely an artifact of pharmacologic doses well in excess of the physiologic range – then such studies are unlikely to advance our understanding or lead to effective therapies.

As discussed in the Introduction, there was good reason to believe that a germline knockout of ghrelin would not produce a decrease in appetite even if the system played a vital role in the adult. Most important in advancing this hypothesis were the studies of Luquet et al.

(Luquet et al., 2005), who used diphtheria toxin (DTX) to ablate NPY/AgRP neurons that were engineered to express the human DTX receptor. When the neurons were ablated in neonatal mice, there was little reduction in food intake, but ablation in adult mice induced starvation. These findings could be explained if compensatory changes ameliorated the effect of ablation when performed in young animals, obscuring the true role of NPY/AgRP neurons. We and many others have reasoned that a similar type of life-long compensation might be obscuring the anorexic effects of ghrelin deficiency when the gene for ghrelin, GOAT or the ghrelin receptor is disrupted in the germline. Therefore, we engineered ghrelin-secreting cells to produce the simian DTX receptor and ablated those cells by injecting DTX in adult mice. The result was identical to the one that we obtained earlier in studies of ghrelin and GOAT germline knockout mice (Zhao et al., 2010a; Li et al., 2012). The ghrelin cell-ablated mice showed no reduction in food intake, and they gained weight as rapidly as controls when placed on a high fat diet. This study therefore rules out neonatal compensation as an explanation for the failure of ghrelin deficiency to alter food intake in mice.

In an attempt to account for the discord between the physiologic effects of ghrelin deletion and the pharmacologic effects of ghrelin injection, we injected doses of ghrelin ranging from 0.01 to 1 mg/kg and measured both food intake and body weight. When we measured the ghrelin level 10 min after the injections, a potential explanation emerged: in both experiments ghrelin stimulated food intake only when the plasma level was raised well above the normal concentration. The highest ghrelin concentration that we have observed in the diurnal rhythm of normal mice is 0.5 to 0.6 ng/ml (Figure 7). Even after a 24 hr fast, plasma ghrelin rises only to 0.9 ng/ml. Yet, when we injected 0.025 mg/kg ghrelin to control mice, we raised the plasma level to 2.7 ng/ml (14-fold above normal) without a significant increase in food intake. When we

doubled the ghrelin dose to 0.05 mg/kg, plasma ghrelin rose by 80-fold to 16 ng/ml and food intake was significantly increased. The feeding response to supraphysiologic ghrelin levels is known to require the ghrelin receptor (Sun et al., 2004), thus indicating that the receptor is far from saturated, even at the highest ghrelin levels that are observed in normal or fasted conditions. The responses of mice over this 5-log range of ghrelin levels is schematized in Figure 13.

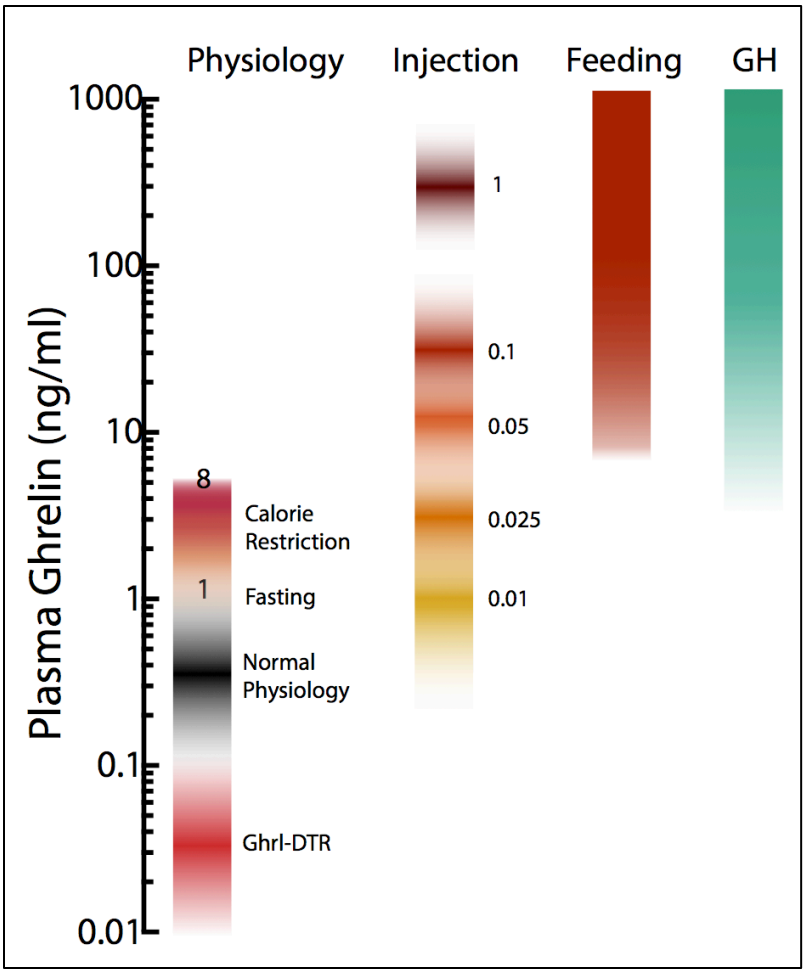

Figure 13. Levels of Ghrelin During Physiologic States and Pharmacologic (Injection) Doses. Ranges in which Feeding and GH responses are seen is Indicated at right.

The requirement for supraphysiologic ghrelin levels to stimulate appetite and raise GH appears to explain the normal food intake and body weight of mice lacking ghrelin or the ghrelin receptor from birth (Sun et al., 2003; Sun et al., 2004; Wortley et al., 2004; Zhao et al., 2010a). Humans may also require supraphysiologic ghrelin levels to stimulate appetite, as shown by Lippl, et al. (Lippl et al., 2012), who infused ghrelin to normal weight volunteers and found no increase in appetite even when plasma ghrelin was raised 4-fold above control values. These

researchers cited 9 previous studies in which increased food intake in humans was observed at doses of ghrelin that raised plasma ghrelin levels many fold above normal.

Thus far, the only documented essential requirement for ghrelin is to maintain life-preserving levels of blood glucose when mice have been depleted of adipose tissue through prolonged severe calorie restriction. In this respect, the ghrelin cell-ablated mice behaved identically to mice that lack GOAT or ghrelin from birth (Figure 8; Zhao et al., 2010a; Li et al., 2012; Goldstein et al., 2011). In control mice, ghrelin levels rise progressively during this period, and GH levels follow. Administration of ghrelin or GH prevents the hypoglycemia, indicating that the essential role of ghrelin is prevention of hypoglycemia during periods of extreme food deprivation (Zhao et al., 2010a; Goldstein et al., 2011).

It should be noted that at the extremes of calorie restriction, when the effect of ghrelin on GH becomes important, one approaches the range of plasma concentrations in which appetite is stimulated. Ghrelin may indeed make mice "hungrier," under these conditions, but when they have been starved to 75% of their initial bodyweight and a plethora of factors are stimulating hunger, ghrelin may simply be gilding the lily, and in practical terms, the drive to eat would be so high that any marginal effect of ghrelin would be extremely difficult to determine.

But the question remains: why *does* ghrelin stimulate appetite at pharmacologic doses if this stimulation is not important *in vivo*? Teleologically, it seems odd that a hormone should have a function that it is not called on to perform in the course of an animal's lifetime. More recent studies on AgRP/NPY neurons by Richard Palmiter's lab as well as Scott Sternson's lab at Janelia Farm may provide an answer. For simplicity, and because the *Agrp* promoter is typically used for genetic targeting, I refer to them simply as AgRP neurons from here on.

Integrating the work of these two scientists provides an interesting insight into the ghrelin paradox because they have taken opposing approaches to study the same population of neurons. Sternson's lab began by optogenetically stimulating AgRP neurons, a situation not unlike that of ghrelin injection, as stimulation of these neurons through the GHSR accounts for ghrelin's stimulation of appetite (Luquet et al., 2007; Wang et al., 2014). They established that stimulation of AgRP neurons in the hypothalamus was indeed sufficient to drive feeding, a result that was contemporaneously demonstrated by Bradford Lowell at Harvard (Aponte et al., 2010; Krashes et al., 2011a). In subsequent studies, Sternson and colleagues mapped the projections from AgRP neurons to other areas of the hypothalamus and brain, and stimulated axon projections from AgRP neurons to those downstream regions independently (Atasoy et al., 2012; Betley et al., 2013). Optogenetics allows for this by first labeling AgRP neurons with channelrhodopsin and then directing optical fibers to a subset of axon projections. Since only AgRP axons are labeled and only those axons located in a particular locus are stimulated, one can specifically stimulate the projections from one population to another.

The results of these studies reveal a complex web of neuronal targets downstream of AgRP stimulation, several of which are independently sufficient to drive feeding, and some of which are not (Betley et al., 2013). Of particular interest are the periventricular nucleus in the hypothalamus (PVH) and the parabrachial nucleus (PBN) in the hindbrain. While both receive axonal inputs from the arcuate AgRP neurons, stimulation of AgRP axons leading to the PVH are sufficient to stimulate hunger, while stimulation of axons leading to the PBN are not (Atasoy et al., 2012). NB: the actions of Agrp neurons on both are inhibitory – this is important, as lesions to the PVH classically cause hyperphagia (Leibowitz et al., 1981).

Palmiter's lab has taken the opposite approach to Sternson's: they ablated the AgRP neurons in adults (Luquet et al., 2005) and, noting that this leads to catastrophic aphagia, endeavored to account for this loss of appetite. Palmiter's first advance came when he characterized the PBN as the critical AgRP target. He found that AgRP neurons constitutively inhibit PBN activity, and their ablation leads to PBN overactivation. When increased PBN activity is suppressed temporarily with GABA agonists after AgRP neuron ablation, adult mice continue to eat a for a critical period, after which they eventually adapt and eat normally in the absence of pharmacologic PBN suppression (Wu et al., 2009). This also occurs if mice are first made obese and can survive the critical period by metabolizing their fat reserves (Wu et al., 2012b). Subsequently, Palmiter and colleagues have mapped both downstream and upstream components for this appetite suppression circuit: excitatory signals from (among other areas) the Nucleus Tractus Solitarius (NTS) stimulate the PBN (Wu et al., 2012a), and the PBN mediates its appetite suppression through projections to the amygdala (Carter et al., 2013). Without GABA-ergic inhibition from the AgRP neurons, this appetite-suppressing circuit is not compatible with life.

Integrating these results suggests an intriguing answer to the ghrelin paradox, as it provides a split between the appetite stimulating effects of AgRP neuron activation (mediated primarily through the PVH), and the appetite suppressing effect of AgRP neuron ablation (mediated through the PBN). If ghrelin only activates AgRP neurons at supraphysiologic levels, then ghrelin injection (like optogenetic stimulation) likely mediates its appetite stimulating effects through AgRP projections to the PVH. Conversely, if physiologic levels are superfluous to maintain AgRP neuron-mediated suppression of PBN output, then loss of ghrelin would not produce a loss of appetite. In summary, a downstream split between the pathways which mediate

the orexigenic effect of AgRP neuron activation and the anorexic effect of AgRP neuron ablation explains how ghrelin can be sufficient but not necessary to stimulate appetite.

Moreover, the complexity revealed by the disparate AgRP neuron projections (some of which do not stimulate appetite) (Betley et al., 2013), as well as more recent studies of divergent functions of the AgRP and NPY neuropeptides themselves (Krashes et al., 2011b), and the myriad factors which activate these neurons (Liu et al., 2012) and mediate their output (Huang et al., 2013; Kong et al., 2012) suggest an web of AgRP neuron interactions coordinately mediating multiple processes, including appetite, energy expenditure, and blood glucose control. Indeed, the PVH itself has been shown to provide excitatory input to AgRP neurons, indicating a feedback loop (Krashes et al., 2014). While the redundancy, complexity, and adaptability of this system has been used to argue in favor of ghrelin being one of many integrated signals, it provides an equally compelling argument for physiologically irrelevant cross-activation of closely interconnected pathways by administration of high doses. It is not sufficient to draw physiologic conclusions from pharmacologic data, especially when those data necessitate concentrations far outside the physiologic range. The role that any one factor plays in the system must be dissected with close attention to physiologic concentration, the conditions in which it is elevated, and the specific consequences of its deletion.

Whether or not ghrelin is integrated into some complex neuronal energy-balance calculation, our data presented here argue that this role is miniscule at best. More importantly, they also show convincingly that inhibition of ghrelin does not produce anorexia. If the human ghrelin axis is similar to mice (which data from other groups suggests is the case (Lippl et al., 2012)), any attempts to inhibit ghrelin in the pursuit of appetite control are therefore unlikely to succeed.

While our results suggest that ghrelin antagonism is unlikely to achieve clinical relevance in treating obesity, ghrelin agonism may have a role in the clinic. Early pharmacologic applications were limited by ghrelin's short half-life, but orally bioavailable ghrelin mimetics with longer half-lives have shown promise in trials for treating cancer cachexia (Garcia et al., 2013). The same therapeutic potential does not appear to be true for anorexics, who seem to be resistant to the appetite stimulating effects of ghrelin agonism (Méquinion et al., 2013).

As far as ghrelin's physiological function: future studies should concentrate on elucidating its preservation of blood glucose during starvation. While this role is now well established, replicated in over 100 experiments in our laboratory and in three separate animal models of ghrelin deficiency, its mechanism remains elusive and further efforts may reveal new insights about the physiology of starvation.

CHAPTER IV: EXPERIMENTAL PROCEDURES

Generation of Mice with DTX-Inducible Ablation of Ghrelin-Producing Cells

All mice (C57BL/6J background unless otherwise indicated) were housed in cages with 12-hr light/12-hr dark cycles. The dark cycle began at 9:00 or 10:00 p.m. All animal experiments were performed with approval of the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center.

To generate Ghrl-DTR mice (Figure S1), we crossed Ghrelin-Cre mice (gift of Jeffrey L Zigman, UT Southwestern) (Engelstoft et al., 2013) to inducible Diphtheria Toxin Receptor (iDTR) mice (Jackson Laboratory; #007900). The latter strain contains a modified *Rosa26* locus in which a flox-inactivated copy of the simian *Hbegf* gene (commonly known as Diphtheria Toxin Receptor gene) (*DTR*) has been inserted (Buch et al., 2005). Expression of Cre recombinase removes a premature stop codon and activates transcription of *DTR*, conferring DTX sensitivity to ghrelin cells. Mice with the genotype *Ghrl-Cre; iDTR$^{f/f}$* or *Ghrl-Cre; iDTR$^{f/+}$* were designated Ghrl-DTR, and littermates without the ghrelin-cre transgene (*iDTR$^{f/+}$* or *iDTR$^{f/f}$*) were used as control mice.

For all experiments, DTX (Sigma, Cat. No. D0564) was dissolved in normal saline at a concentration of 1.0-1.2 μg/ml, and an injection of 200 μl was administered intraperitoneally to all mice, including controls. This corresponds to a dose of 8-10 ng DTX/g body weight. Of all mice used in these studies, 91% experienced a robust ablation of ghrelin cells after a single DTX injection; 9% showed no response to DTX administration. Partial responses were not observed in any of the injected mice.

Diets

The chow diet (Teklad Mouse/Rat Diet 7002; Harlan Teklad Global Diets) contains 3.0 kcal/g of metabolizable energy, of which 18% of calories are from fat, 49% from carbohydrates, and 33% from protein. For high fat diet (HFD) studies, mice were fed a diet containing 45% calories from fat. The diet was composed of 24 gm% fat, 41 gm% carbohydrate, and 24 gm% protein (Research Diets; Cat No. D12451). Total body fat was measured by NMR (Bruker Minispec mq7.5 NMR analyzer).

Calorie Restriction

Calorie restriction was carried out as described by Zhao, et al. (Zhao et al., 2010a). One week before initiation of calorie restriction, 8-week-old male control and Ghrl-DTR littermates were placed in individual cages and fed the chow diet ad libitum. During this week, food intake was monitored to determine the average daily food consumption of each individual mouse. On day 0, all mice received a single IP injection of 8-10 ng/g DTX and were subjected to 60% calorie restriction such that each mouse was fed 40% of its average food consumption daily at 6:00 p.m. Body weight and blood glucose were measured daily at 5:30 p.m. before feeding.

Administration of Ghrelin

Mice were briefly anesthetized with isoflurane and injected subcutaneously with ~ 0.1 ml of 0.15 M sodium chloride containing the indicated concentration of ghrelin (Genscript, Cat No. RP10781-5).

Blood Collection and Hormone Measurements

For measurements in which mice were kept alive (Figures 3-6, S2, and S3), 10-30 μl of tail vein blood was collected. For measurements in which mice were sacrificed (Figures 1, S1, and S4), mice were anesthetized with isoflurane, after which 500-700 μl blood was drawn from the inferior vena cava (Figures 1, and S1) or the orbit (Figure S4). Blood samples were stored in EDTA-coated tubes containing p-hydroxymercuribenzoic acid (final concentration, 1 mM). Plasma was separated into two aliquots and stored at -80° C: one for insulin or GH measurements and the other for ghrelin/des-acyl ghrelin measurements, the latter stored in HCl (final concentration, 0.1 M). Ghrelin and des-acyl ghrelin were measured with immunoassay kits from Cayman Chemical that distinguish both forms of the peptide (Cat. No. 10006307 and No. 10008953). Insulin was measured with an ELISA kit from Crystal Chem (Cat No. 90080). GH was measured with an ELISA kit from Cayman Chemical (Cat No. 589601).

Quantitative Real-Time PCR

Total RNA was isolated from mouse kidney, liver, duodenum, and whole stomach, after which the mRNAs were subjected to real-time PCR using primers for mouse cyclophilin, ghrelin, and chromogranin A as previously described (Zhao et al., 2010b). Additional primers used (forward and reverse) were as follows: mouse TNFα, 5′-CTGAGGTCAATCTGCCCAAGTAC-3′ and 5′-CTTCACAGAGCAATGACTCCAAAG-3′; cre recombinase, 5′-GGCCCAAATGTTGCTGGAT-3′ and 5′-TGTTCGCGATTATCTTCTATATCTTCA-3′; simian HBEGF, 5′-AGGCAAGGGACTAGGGAAGA-3′ and

5′-CCACCACAGCCAGGATAGTT-3′. All reactions were done in triplicate. The relative amount of all mRNAs was calculated using the comparative threshold cycle (C_t) method. Cyclophilin mRNA was used as the invariant control.

Histology

Stomachs were fixed for 20-48 hr in 4% (v/v) paraformaldehyde in PBS. The fixed tissues were embedded in paraffin and sectioned at 4 μm. For immunohistochemistry, slides were pretreated with 0.3% (v/v) hydrogen peroxide for 15 min at room temperature, followed by antigen retrieval for 10 min at 98°C in Retrievagen A buffer (BD Biosciences). The slides were then incubated for 1 hr at room temperature in 10% (v/v) normal goat serum (NGS; Vector Labs) in PBS, followed by overnight incubation at 4°C with anti-ghrelin antiserum (1:1000 dilution in 10% NGS; Phoenix Pharmaceuticals, Code H-031-31, Lot no. 01241-3) or anti-chromogranin A antiserum (1:400 in 10% NGS; Abcam, Code ab15160, Lot no. GR121602-3). Slides were then incubated for 1 hr at room temperature with anti-rabbit antiserum (1:500 in 10% NGS; Jackson Immunoresearch), followed by 60 min at room temperature in Streptavidin-HRP conjugate (1:750 in 10% NGS; Invitrogen). Activity was developed for 1 min with an AEC substrate kit (Invitrogen), counterstained with hematoxylin, and mounted using Aqua-Polymount (Polysciences, Inc.).

CHAPTER V: REFERENCES

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