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I, Kristy M. Heppner, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Neuroscience/Medical Science Scholars Interdisciplinary.

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Acylation state determines the action of ghrelin on energy and glucose metabolism

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Acylation state determines the action of ghrelin on energy and glucose metabolism

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General Abstract

Ghrelin is a gastrointestinal hormone that activates the ghrelin receptor (GHSR) in hypothalamic neurons to promote feeding and peripheral lipid storage. Ghrelin contains a unique acyl modification that is esterified to the peptide molecule. The recent discovery of the ghrelin acylating enzyme, ghrelin O-acyltransferase (GOAT), has provided a deeper understanding of the acylation process and actions of acyl ghrelin (AG) versus des-acyl ghrelin (dAG). Our group previously demonstrated that GOAT utilizes various dietary fatty acids (FAs) as a substrate for ghrelin acylation. In the current study, we use both in vitro and in vivo approaches to demonstrate that ghrelin action on GHSR activation, food intake, body weight and fat mass can be modified by altering the FA side-chain length of the ghrelin peptide molecule. Additionally, we find that AG and dAG act in the central nervous system to increase glucose-stimulated plasma insulin levels indicating that this function of ghrelin is independent of its acylation state. Finally, we combine genetic and pharmacological approaches to demonstrate that the regulation of glucose tolerance by GOAT action is dependent on calorie restriction and age. Collectively, these data systematically dissect the function of ghrelin acylation in regulating energy and glucose metabolism.
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List of Abbreviations
AG  Acyl ghrelin (C8-acylated ghrelin)
AgRP  Agouti-related peptide
AMPK  AMP-activated protein kinase
AN  Anorexia nervosa
ANOVA  Analysis of variance
APT1  Acyl-protein thioesterase 1
ARC  Arcuate nucleus
BAT  Brown adipose tissue
BBB  Blood-brain-barrier
BuChE  Butyrylcholinesterase
BW  Body weight
CaMKK2  Calcium/calmodulin kinase kinase 2
CNS  Central nervous system
dAG  des-acyl ghrelin
DIO  Diet-induced obese
DRD2  Dopamine receptor subtype-2
EGP  Endogenous glucose production
ELISA  Enzyme-linked immunosorbent assay
FA  Fatty acid
FI  Food intake
FM  Fat mass
GH  Growth hormone
GHSR  Growth hormone secretagogue receptor
GIR  Glucose infusion rate
GLP-1  Glucagon-like peptide 1
GOAT  Ghrelin O-acyltransferase
GPCR  G-protein coupled receptor
GSIS  Glucose stimulated insulin secretion
GTT  Glucose tolerance test
HFD  High fat diet
ICV  Intracerebroventricular
IP  Inositol phosphate
IP Intraperitoneal
IP$_3$  Inositol triphosphate
IV intravenous
LPS  Lipopolysaccharide
MBOAT Membrane-bound O-acyltransferase
NPY Neuropeptide Y
PVN  Paraventricular nucleus
PWS  Prader-Willi syndrome
RIA Radioimmunoassay
SC subcutaneous
SST5  Somatostatin receptor 5
T2DM  Type 2 diabetes mellitus
TM Transmembrane
UCP Uncoupling protein
VMH  Ventral medial hypothalamus
WAT White adipose tissue
WT Wild-type
CHAPTER 1

General Introduction and Background
Public Health Perspectives: Obesity and Diabetes

Obesity is a major public health concern that is affecting nations worldwide (Misra and Khurana 2008; Flegal, Carroll et al. 2012). The rise in obesity has led to an increased occurrence of co-morbid diseases including cardiovascular disease, cancer, and type 2 diabetes mellitus (T2DM) (Guh, Zhang et al. 2009; Vucenik and Stains 2012). T2DM is characterized by insulin resistance of insulin-responsive tissues such as liver, muscle and adipose tissue. As the disease progresses, decreased insulin secretion from the β-cells can occur (Zimmet, Alberti et al. 2001). Treating obesity-related morbidities has put a major strain on health care systems around the world (Visscher and Seidell 2001). Treatments for T2DM alone has cost the US health care system billions of dollars annually (Ariza, Vimalananda et al. 2010). Understanding the underlying mechanisms that regulate energy and glucose metabolism can lead to the development of more effective therapies to alleviate obesity and T2DM. The gastrointestinal hormone, ghrelin, has been a popular target because of its obesogenic and diabetogenic properties. Ghrelin levels in obese patients as well as in T2DM patients are reduced, but whether this is a risk factor or associative factor is unclear (Tschop, Weyer et al. 2001; McLaughlin, Abbasi et al. 2004). In order to develop effective treatments that target ghrelin activity, further research is required to gain a thorough understanding of ghrelin action on energy and glucose metabolism.
**Ghrelin: Overview**

Ghrelin is a 28-amino acid peptide that was discovered as the endogenous ligand for the growth hormone secretagogue receptor-1a (GHSR) and was initially noted for its growth hormone (GH) releasing property (Kojima, Hosoda et al. 1999). Ghrelin possesses a unique post-translational modification where an $n$-octanoic acid is esterified to the serine3 residue of the peptide molecule. The presence of a fatty acid (FA) side-chain attached to the ghrelin peptide is required for full agonism of GHSR (Kojima, Hosoda et al. 1999). The fact that ghrelin was isolated from stomach extracts together with the finding that GHSR is co-expressed in key hypothalamic neurons involved in regulating energy metabolism (Willesen, Kristensen et al. 1999) suggested that ghrelin may act to link peripheral metabolic status with CNS regulation of energy metabolism. A number of groups confirmed that ghrelin is an important regulator energy balance. Tschöp *et al.* revealed that chronic peripheral as well as chronic intracerebroventricular (icv) administration of ghrelin induces adiposity in rodents (Tschop, Smiley et al. 2000). Wren *et al.* demonstrated that acute peripheral as well as icv administration of ghrelin increases acute food intake in rats (Wren, Small et al. 2000). Nakazato *et al.* showed that ghrelin activates hypothalamic neuropeptide Y (NPY) and Agouti-related peptide (AgRP) neurons to elicit its orexigenic action (Nakazato, Murakami et al. 2001). Together, these initial findings highlighted ghrelin as a key component of the gut-brain axis in regulating energy metabolism.

The regulation of energy balance and glucose homeostasis are intimately linked (Muoio and Newgard 2006), and therefore, much effort was dedicated to exploring the role of
ghrelin in regulating glucose homeostasis. Ghrelin and its receptor are both expressed in pancreatic islet cells, suggesting that ghrelin may have paracrine or autocrine action in the pancreas (Kageyama, Funahashi et al. 2005). Accordingly, ghrelin inhibits glucose-stimulated insulin release in isolated islet and β-cells (Dezaki, Hosoda et al. 2004; Dezaki, Kakei et al. 2007; Qader, Hakanson et al. 2008). Furthermore, acute peripheral administration of ghrelin inhibits glucose-stimulated insulin secretion leading to an impairment in glucose tolerance in both rodents (Reimer, Pacini et al. 2003) (Dezaki, Hosoda et al. 2004; Cui, Ohnuma et al. 2008) and humans (Broglio, Arvat et al. 2001; Broglio, Gottero et al. 2004; Tong, Prigeon et al. 2010). Taken as a whole, these data suggest that ghrelin antagonism is a promising therapy for treating obesity and T2DM.

**Ghrelin processing**

The human pre-proghrelin gene (GHRL) is located on chromosome 3 at position q25-26 and is comprised of 4 exons (Smith, Van der Ploeg et al. 1997). The functional ghrelin peptide is encoded by exon 1 and 2. The amino acid sequence of ghrelin is highly conserved among species and rat and human ghrelin sequences differ by only 2 amino acids (Kojima, Hosoda et al. 1999). During the first step of processing, the signal sequence of pre-proghrelin is cleaved leaving a 94 amino acid segment, proghrelin. During post-translational processing, proghrelin undergoes acylation and cleavage by separate enzymes. The order in which these post-translational processes occur is not clear, although some evidence suggests that acylation occurs before the final cleavage step (Takahashi, Ida et al. 2009). The enzyme responsible for ghrelin acylation is
ghrelin O-acyltransferase (GOAT). GOAT esterifies a medium-chain fatty acid (most commonly n-octanoic acid) to the serine3 residue of the proghrelin peptide molecule. Cleavage of proghrelin is catalyzed by prohormone convertase PC1/3, which produces an N-terminal 28-amino acid sequence (ghrelin) and a C-terminal peptide called obestatin (Zhu, Cao et al. 2006). Initial reports indicated that obestatin possessed anorexigenic properties by acting on GPR39 (Zhang, Ren et al. 2005). These data were not able to be replicated by other groups (Seoane, Al-Massadi et al. 2006; Holst, Egerod et al. 2007; Nogueiras, Pfluger et al. 2007) and therefore, the function of obestatin remains controversial.

**Ghrelin secretion**

Ghrelin is predominately secreted from the X/A-like cells within the gastric oxyntic mucosa (Date, Kojima et al. 2000). Lower levels of ghrelin are produced in other tissues including the duodenum, jejunum, ileum, colon, hypothalamus, pituitary, pancreas, thyroid, and kidney (Mori, Yoshimoto et al. 2000; Korbonits, Bustin et al. 2001; Gnanapavan, Kola et al. 2002; Wierup, Svensson et al. 2002; Cowley, Smith et al. 2003; Mondal, Date et al. 2005; Raghay, Garcia-Caballero et al. 2006). Two main forms of ghrelin are found in circulation: an acylated form and a more abundant des-acyl form. The factors that regulate ghrelin secretion are not entirely clear. Total circulating ghrelin levels are low in obese humans (Tschop, Weyer et al. 2001) and increase with diet induced weight loss (Hansen, Dall et al. 2002). The reason that ghrelin levels are lower in obese subjects is not fully understood but may reflect a compensatory adaptation that aims to reduce a hunger stimulus. In contrast to low ghrelin levels in the
obese state, ghrelin levels are increased in disease states of negative energy balance such as anorexia nervosa (Ariyasu, Takaya et al. 2001) and cancer cachexia (Shimizu, Nagaya et al. 2003; Hanada, Toshinai et al. 2004). These high ghrelin levels decline upon weight gain (Otto, Cuntz et al. 2001). Plasma ghrelin levels are increased in patients with Prader-Willi syndrome (PWS) (Cummings, Clement et al. 2002), a genetic disorder characterized by severe hyperphagia, short stature, and mental retardation. The characteristics of PWS phenotype are thought to be a consequence of developmental abnormalities in hypothalamic regions that regulate appetite; however, it is unclear as to what mechanisms are responsible for the increased ghrelin levels in these patients. Low plasma ghrelin levels are associated with insulin resistance in humans (Poykko, Kellokoski et al. 2003; McLaughlin, Abbasi et al. 2004). The lower levels are due to decreased des-acyl ghrelin (dAG), whereas acyl ghrelin (AG) levels are actually increased in obese individuals with T2DM (Rodriguez, Gomez-Ambrosi et al. 2012). However, the reason for the correlation between ghrelin levels and insulin resistance and T2DM is not clear.

One factor that clearly regulates ghrelin secretion is feeding. Initial reports found that total circulating ghrelin levels increase during fasting (Tschop, Smiley et al. 2000). Furthermore, plasma ghrelin levels surge prior to consumption of a meal (Cummings, Purnell et al. 2001) and decrease following ingestion of nutrients (Tschop, Wawarta et al. 2001) indicating that ghrelin may be involved in meal initiation. The macronutrient composition of the meal also determines the magnitude of ghrelin suppression with proteins being most effective and lipids being least effective to suppress postprandial
The creation of sophisticated ghrelin assays enabled researchers to more accurately measure AG versus dAG levels. Under fed conditions in humans, ghrelin levels fluctuate in a unique pattern where both AG and dAG increase immediately prior to a meal and decrease following nutrient ingestion (Liu, Prudom et al. 2008). During prolonged fasting, total ghrelin levels are similar to total ghrelin levels in the fed state. However, the des-acyl form of ghrelin increase to peak pre-prandial levels, whereas AG levels remain at low levels similar to those seen postprandially (Liu, Prudom et al. 2008). The activity of butyrylcholinesterase (BuChE), a ghrelin deacylation enzyme, was not altered due to fasting indicating that the decrease in AG is not a result of increased deacylation. Together, these data indicated that ghrelin secretion is regulated independently of ghrelin acylation. Shortly after these findings, the ghrelin acylating enzyme was discovered which enabled researchers to further understand the regulation of ghrelin secretion versus ghrelin acylation as well as understand the endogenous roles of AG versus dAG.

**Ghrelin acylation by ghrelin O-acyltransferase (GOAT)**

The enzyme that catalyzes the acylation of ghrelin is ghrelin O-acyltransferase (GOAT) (Gutierrez, Solenberg et al. 2008; Yang, Brown et al. 2008). GOAT is located in the endoplasmic reticulum of the cell and is a member of the family of proteins called membrane-bound O-acyltransferases (MBOATs) and is also known as MBOAT4. Similar to ghrelin gene expression, GOAT mRNA is predominately found in the stomach and pancreas (Gutierrez, Solenberg et al. 2008; Yang, Brown et al. 2008). Lower levels
have been found in the intestines, adrenal, kidney, pituitary, and hypothalamus (Sakata, Yang et al. 2009; Lim, Kola et al. 2011; Gahete, Cordoba-Chacon et al. 2012). The expression of GOAT is influenced by a number of metabolic factors (reviewed in (Romero, Kirchner et al. 2010)). For example, gastric GOAT expression decreases during short-term fasting and long-term calorie restriction (Kirchner, Gutierrez et al. 2009; Reimer, Maurer et al. 2010), whereas other groups have found that short term fasting and long-term calorie restriction increases GOAT expression (Gonzalez, Vazquez et al. 2008; Gahete, Cordoba-Chacon et al. 2010). One study demonstrated that obese patients with T2DM have higher GOAT levels in visceral adipose tissue (Rodriguez, Gomez-Ambrosi et al. 2012). Taken together, metabolic status regulates GOAT expression, although a further understating of how metabolic status regulates GOAT expression warrants further clarification.

The most abundant form of endogenously produced AG is octanoyl (C8) ghrelin. However, GOAT is able to use fatty acids (FAs) of various side-chain lengths ranging from C2-C16 as acyl substrates (Hosoda, Kojima et al. 2003; Gutierrez, Solenberg et al. 2008; Ohgusu, Shirouzu et al. 2009). Furthermore, GOAT can utilize lipids from the diet as a substrate for ghrelin acylation (Nishi, Hiejima et al. 2005; Kirchner, Gutierrez et al. 2009). A number of in vitro experiments have investigated GHSR activation by these ghrelin isoforms with varying acyl side-chain lengths (Bednarek, Feighner et al. 2000; Matsumoto, Hosoda et al. 2001). These studies demonstrate that acylation of ghrelin with a side-chain shorter than 4 carbons significantly reduces the potency of GHSR activation. An in vivo study explored the effect of an endogenously produced ghrelin
isoform, C10-acylated ghrelin on GH secretion in rats (Hosoda, Kojima et al. 2003). The C10-acylated isoform had a similar potency to induce GH secretion as compared to C8-acylated ghrelin. Whether modulating the FA side-chain will alter ghrelin action on energy metabolism has not been investigated. Furthermore, GHSR independent action of ghrelin acylated with different FAs has not been examined.

**The growth hormone secretagogue receptor (GHSR)**

The ghrelin receptor was discovered using synthetic growth hormone secretagogues and was named for its function to promote GH secretion (Howard, Feighner et al. 1996). It was renamed the ghrelin receptor after the discovery of its endogenous ligand (Kojima, Hosoda et al. 1999) but is still commonly referred to as GHSR. The human GHSR gene is located on chromosome 3 at position q26.2 and encodes a 7-transmembrane G-protein coupled receptor (7TM GPCR) (Petersenn, Rasch et al. 2001). The gene consists of two exons; the first exon encodes TM1−TM5, and the second exon encodes TM6−TM7. Two mRNA splice variants have been identified: GHSR-1a and GHSR-1b (McKee, Palyha et al. 1997). GHSR-1a is a full 7TM GPCR and has high affinity binding for ghrelin. GHSR-1b is derived from exon 1, and encodes only five of the seven predicted TM domains. GHSR-1b does not bind ghrelin and was initially thought to be physiologically inactive. Some reports show that GHSR-1b modulates GHSR-1a action; however, the exact physiological function of GHSR-1b is not known (Chu, Chow et al. 2007; Leung, Chow et al. 2007).

The highest expression of GHSR is in the somatotrophic pituitary cells of the anterior lobe of the pituitary, as well as the arcuate nucleus (ARC) of the hypothalamus.
(Howard, Feighner et al. 1996). This is consistent with the role of ghrelin in regulating GH release and energy balance. Lower expression is found in peripheral tissues including pancreas, adrenal, myocardium, thyroid, spleen, kidney, skeletal muscle and adipose tissue (Gnanapavan, Kola et al. 2002; Lin, Saha et al. 2012). GHSR is also expressed in other hypothalamic nuclei including the ventral medial hypothalamus (VMH) and paraventricular nucleus (PVN). Consistent with ghrelin action on learning and memory (Diano, Farr et al. 2006) as well as food reward (Perello and Zigman 2012), GHSR expression is found in the CA2 and CA3 region of the hippocampus and the ventral tegmental area (Guan, Yu et al. 1997; Zigman, Jones et al. 2006). GHSR expression is regulated by a number of different factors. Fasting upregulates GHSR expression particularly in the hypothalamus (Kim, Yoon et al. 2003; Petersen, Woldbye et al. 2009). Diet-induced obesity (DIO) decreases hypothalamic expression of GHSR (Briggs, Enriori et al. 2010), which could be the reason that animals fed a high fat diet (HFD) become resistant to ghrelin induced hyperphagia (Briggs, Enriori et al. 2010; Perez-Tilve, Heppner et al. 2011). GHSR expression in white and brown adipose tissue increases as animals age, and lack of GHSR signaling has been suggested to contribute to the leaner phenotype in aged Ghsr−/− mice (Lin, Saha et al. 2012).

The ghrelin receptor is unique in that it retains high basal constitutive signaling (~50%) (Holst, Cygankiewicz et al. 2003). Many early reports indicated that the presence of an acyl side-chain attached to the ghrelin molecule was required for ghrelin to bind and activate GHSR (Kojima, Hosoda et al. 1999; Bednarek, Feighner et al. 2000; Matsumoto, Hosoda et al. 2001). However, later studies showed that dAG is a weak
agonist of GHSR (Bednarek, Feighner et al. 2000) and high concentrations of dAG have been reported to activate GHSR (Heppner et al., in preparation)(Gauna, van de Zande et al. 2007). GHSR couples to the Ga_q/11 subunit leading to activation of phospholipase C which leads to increased inositol triphosphate (IP_3) accumulation and intracellular calcium release in the pituitary and hypothalamus (Howard, Feighner et al. 1996; Holst, Holliday et al. 2004). Therefore, inositol phosphate (IP) mediated calcium release is commonly used as a read-out of GHSR activation. Several kinases and transcription factors are then activated downstream of the Ga_q/11 protein coupling. In the hypothalamus, ghrelin activates AMP-activated protein kinase (AMPK) which leads to an increase in NPY/AgRP expression and subsequent increase in food intake (Kohno, Sone et al. 2008; Lopez, Lage et al. 2008). Calcium/calmodulin kinase kinase 2 (CaMKK2) has been demonstrated to activate AMPK in the hypothalamus (Anderson, Ribar et al. 2008). CaMKK2 is involved in mediating the orexigenic action of ghrelin as CaMKK2 null mice do not have increased food intake in response to peripherally administered ghrelin (Anderson, Ribar et al. 2008). In addition to Ga_q/11 coupling, GHSR signalling via Ga_i/o and Ga_s coupling has also been reported (Kohno, Gao et al. 2003; Carreira, Camina et al. 2004; Jiang, Betancourt et al. 2006; Dezaki, Kakei et al. 2007; Kern, Albarran-Zeckler et al. 2012). The factors that determine which G-protein signalling system GHSR couples to are not clear, but may have to due with tissue specificity as well as the influence of other GPCRs and ligands present. For example, GHSR in pancreatic islets is able to heterodimerize with the somatostatin receptor 5 (SST5) which shifts GHSR coupling from Ga_q/11 to Ga_i/o (Park, Jiang et al. 2012). The ability of GHSR to heterodimerize with other G-protein coupled receptors (GPCRs) is

Understanding GHSR heterodimerization with other GPCRs and downstream G-protein coupling may help to elucidate how these systems interact to regulate energy homeostasis. After ghrelin binds and activates GHSR, the receptor undergoes rapid homologous desensitization via clathrin-mediated endocytosis and is then recycled back to the plasma membrane (Camina, Carreira et al. 2004). In summary, changes in gene expression, the constitutive activity of the receptor, and heterodimerization with other GPCRs are all factors that have made it challenging to characterize the specific endogenous role of GHSR.

**Ghrelin deacylation and proteolysis**

Although much effort was put into identifying the ghrelin acylating enzyme, the enzymes and pathways involved in ghrelin deacylation are poorly understood (Satou, Nakamura et al. 2011). Due to original evidence that suggested that dAG was an inactive peptide (Kojima, Hosoda et al. 1999), not much focus was put on the ghrelin delipidation enzymes. However, a number of studies have highlighted a wide variety of physiological actions of dAG (Bulgarelli, Tamiazzo et al. 2009; Delhanty, Huisman et al. 2011). Although much effort was put into identifying the ghrelin acylating enzyme, the enzymes and pathways involved in ghrelin deacylation are poorly understood (Satou, Nakamura et al. 2011). Due to original evidence that suggested that dAG was an inactive peptide (Kojima, Hosoda et al. 1999), not much focus was put on the ghrelin delipidation enzymes. However, a number of studies have highlighted a wide variety of physiological actions of dAG (Bulgarelli, Tamiazzo et al. 2009; Delhanty, Huisman et al. 2011).
2013; Porporato, Filigheddu et al. 2013), and therefore, understanding the deacylation process could be a critical determinant for understanding dAG function. The acyl side-chain of ghrelin is rapidly cleaved by circulating enzymes making careful handling and treatment of blood samples essential for accurate measurement of AG and dAG (Hosoda, Doi et al. 2004; Liu, Prudom et al. 2008). Ghrelin deacylation differs highly among species. The half-life of AG in human serum is approximately 240 minutes whereas it is only 30 minutes in rat serum (De Vriese, Gregoire et al. 2004). The enzymes involved in ghrelin deacylation differ between rodents and humans. BuChE is one of the major enzymes responsible for ghrelin deacylation in humans, whereas liver carboxylesterase is responsible for ghrelin deacylation in rodents (De Vriese, Gregoire et al. 2004). Recently, acyl-protein thioesterase 1 (APT1)/lysophospholipase I (APT1) was identified as a ghrelin deacylation enzyme in bovine serum (Satou, Nishi et al. 2010). APT1 mRNA is suppressed in the presence of lipopolysaccharide (LPS) suggesting that deacylation may be regulated during septic inflammation. Following deacylation, ghrelin is broken down into smaller peptide fragments by endopeptidases and is hydrolyzed by carboxypeptidases (De Vriese, Gregoire et al. 2004). The specific enzymes involved in this processes as well as the physiological role of these smaller peptide fragments are unknown.

**Acyl ghrelin (AG) regulation of food intake**

Acyl ghrelin has potent orexigenic action in both rodents (Wang, Saint-Pierre et al. 2002) (Wren, Small et al. 2000; Date, Shimbara et al. 2006) and humans (Wren, Seal et al. 2001; Druce, Neary et al. 2006). AG increases food intake by activating brain areas
that regulate homeostatic feeding. Specifically, ghrelin acts on GHSR located on orexigenic NPY and AgRP neurons in the ARC (Nakazato, Murakami et al. 2001; Wang, Saint-Pierre et al. 2002; Chen, Trumbauer et al. 2004). In addition to acting in brain regions that regulate homeostatic feeding, ghrelin also activates reward centers of the brain to increase hedonic feeding (Malik, McGlone et al. 2008). Peripherally administered AG crosses the blood-brain-barrier (BBB) (Banks, Tschop et al. 2002) and can passively and rapidly diffuse through fenestrated capillaries of the median eminence, a circumventricular organ, to activate NPY neurons in the ventromedial ARC (Schaeffer, Langlet et al. 2013). Passage of ghrelin across the BBB is increased during fasting (Schaeffer, Langlet et al. 2013) and decreases in DIO animals (Banks, Burney et al. 2008). Consistently, DIO animals are resistant to the orexigenic effect of ghrelin. The impaired ability of ghrelin to activate NPY/AgRP neurons in the ARC of DIO animals may be attributed to decreased passage of ghrelin across the BBB or to decreased expression of hypothalamic GHSR and Npy/Agpr in DIO animals (Briggs, Enriori et al.). In addition to crossing the BBB, ghrelin mediates its orexigenic action through activation of GHSR on gastric vagal afferent nerves (Asakawa, Inui et al. 2001; Date, Murakami et al. 2002; le Roux, Neary et al. 2005). This leads to activation of neurons in the nucleus of the solitary tract (NTS), which promotes release of noradrenaline release in ARC (Date, Shimbara et al. 2006).

**Acyl ghrelin regulation of adiposity**

In addition to regulating short-term food intake, AG induces adiposity and body weight gain through multiple mechanisms including modulation of food intake, energy
expenditure, nutrient partitioning, adipocyte metabolism, and locomotor activity.

Chronic icv infusion in rats consistently induces hyperphagia (Tschop, Smiley et al. 2000; Theander-Carrillo, Wiedmer et al. 2006; Perez-Tilve, Heppner et al. 2011; Pfluger, Castaneda et al. 2011). However, chronic AG treatment can induce adiposity in the absence of hyperphagia (Theander-Carrillo et al. 2006) suggesting that ghrelin's regulation of food intake and adiposity are through independent pathways. HFD exposure impairs the orexigenic but not the adipogenic action of AG (Perez-Tilve, Heppner et al. 2011) which provides further support that separate hypothalamic circuits regulate the orexigenic and adipogenic effects of ghrelin.

AG acts centrally to decrease energy expenditure (Asakawa, Inui et al. 2001) in part through suppression of sympathetic nerve activity in brown adipose tissue (BAT) (Yasuda, Masaki et al. 2003). In terms of nutrient partitioning, AG causes a shift in macronutrient utilization to favor carbohydrates rather than fats as a major energy source (Tschop, Smiley et al. 2000; Pfluger, Kirchner et al. 2008). Additionally, AG acts through central mechanisms to alter adipocyte metabolism resulting in the inhibition of lipid oxidation and an increase in lipogenesis and triglyceride uptake in white adipose tissue (Theander-Carrillo, Wiedmer et al. 2006). Chronic icv treatment with AG increases the expression of enzymes in white adipose tissue (WAT) including lipoprotein lipase, acetyl-CoA carboxylase α, fatty acid synthase, stearoyl-CoA desaturase–1 whereas expression of carnitine palmitoyl transferase–1α is decreased. AG decreases brown adipocyte metabolism by downregulating uncoupling protein 1 and 3 (UCP1 and UCP3) (Theander-Carrillo, Wiedmer et al. 2006). Lastly, AG promotes a
positive energy balance by decreasing spontaneous locomotor activity (Tang-
Christensen, Vrang et al. 2004; Pfluger, Castaneda et al. 2011). Collectively, AG acts
through multiple mechanisms to promote a positive energy balance resulting in
increased adiposity.

**Acyl ghrelin regulation of insulin secretion**

In addition to regulating energy balance, AG regulates glucose metabolism in part
through the regulation of insulin secretion. Ghrelin and its receptor are both expressed
in pancreatic islet cells (alpha, beta and epsilon) suggesting regulation of islet cell
function (Date, Nakazato et al. 2002; Volante, Allia et al. 2002; Wierup, Svensson et al.
results using *in vitro* models have made it difficult to confirm the role ghrelin has on
insulin secretion. Treatment of rat islets with AG increased glucose-induced insulin
release (Date, Nakazato et al. 2002), yet other groups find that higher concentrations of
AG inhibited glucose-induced insulin release from rat islets (Colombo, Gregersen et al.
2003; Qader, Hakanson et al. 2008) as well as from INS-1 cells (Wierup, Yang et al.
2004). In mouse islets, AG at low doses inhibited insulin secretion, whereas high doses
enhanced insulin secretion (Qader, Hakanson et al. 2008). It seems as though some of
these discrepancies describing AG’s regulation of insulin secretion might be explained
by dose-dependent effects of AG or may depend on the experimental condition.

GHMR heterodimerization with other GPCRs may also lead to differences in
experimental results concerning ghrelin’s action on insulin secretion. GHMR
heterodimerizes with SST5 in β-cells (Park, Jiang et al. 2012). This heteromer formation is influenced by energy status as well as the ratio of ghrelin:somatostatin present. Moreover, heterodimerization with SST5 shifts GHSR coupling from Ga\textsubscript{q/11} to Ga\textsubscript{i/o} (Park, Jiang et al. 2012). Coupling to Ga\textsubscript{q/11} typically results in increased insulin secretion whereas Ga\textsubscript{i/o} is inhibitory on insulin secretion (Sharp 1996; Gilon and Henquin 2001). Therefore, the shift in G-protein coupling may be one of the reasons that AG can either inhibit or stimulate insulin secretion. Therefore, depending on the cell type used, other GPCRs present and the concentration of AG applied to the cells may influence whether AG enhances or inhibits insulin secretion.

The fact that GHSR is a constitutively active receptor (Holst, Cygankiewicz et al. 2003; Holst, Holliday et al. 2004; Petersen, Woldbye et al. 2009) makes it even more difficult to determine the effects of AG on insulin secretion. When a GHSR antagonist is applied to isolated rat islets or perfused pancreas (Dezaki, Sone et al. 2006), glucose-induced insulin release is enhanced, although it is speculated that the increased insulin levels could be a result of the antagonist blocking the constitutive activity of GHSR, rather than blocking any potential GHSR-independent action of the ghrelin molecule.

Reports in rodent models have been more consistent regarding the action of AG on insulin secretion. Although one study suggested that AG enhances insulin secretion in rats (Lee, Wang et al. 2002), most rodent data support ghrelin having an inhibitory effect on insulin secretion. AG administered intravenously (iv) to mice is able to attenuate glucose-induced insulin secretion (Reimer, Pacini et al. 2003). Consistent with this
finding, GH-deficient mice experienced decreased glucose-stimulated insulin levels after intraperitoneal (ip) injection of AG indicating the effects of AG on insulin secretion are independent of GH (Dezaki, Hosoda et al. 2004). A study in rats demonstrated that AG infused into the portal vein results in a reduction in glucose-stimulated insulin secretion; however, AG infusion into the femoral vein did not have an inhibitory effect on insulin secretion (Cui, Ohnuma et al. 2008). Additionally, when these rats received hepatic vagotomy or co-infusion with atropine methyl bromide, they did not experience the inhibitory effect of AG on glucose-stimulated insulin secretion indicating that hepatic portal system and the vagus nerve are required to induce AG's inhibitory effect on insulin secretion (Cui, Ohnuma et al. 2008).

A limited number of studies describing the effect of AG on insulin secretion have been reported in humans. AG administered to healthy humans decreases fasting insulin levels (Broglio, Arvat et al. 2001; Broglio, Gottero et al. 2004), although another group reported no change (Lucidi, Murdolo et al. 2005). Tong et al. (Tong, Prigeon et al. 2010) demonstrated that AG inhibits intravenous glucose-stimulated insulin secretion in healthy humans. In summary, the in vitro reports are the most conflicting regarding the physiological action of AG on insulin secretion. The majority of studies in rodents suggest that AG inhibits insulin secretion in vivo. The few studies reporting AG effects on insulin levels in humans show either decreased or no effect on fasting insulin whereas iv glucose-stimulated insulin secretion is decreased by AG.
Mechanisms mediating the action of AG on insulin secretion

Several mechanisms whereby AG regulates insulin secretion have been proposed. Ghrelin is expressed in pancreatic islets and therefore may exert its effects on insulin secretion through paracrine or autocrine action on the β-cell (Date, Nakazato et al. 2002; Volante, Allia et al. 2002; Wierup, Svensson et al. 2002). In the β-cell, AG increases delayed rectifier K⁺ currents and inhibits glucose-induced increases in intracellular calcium, leading to decreased insulin release (Dezaki, Hosoda et al. 2004). AG can GHSR coupling to Ga₁/0 to attenuate glucose-induced increases in intracellular calcium which reduces insulin release from the β-cells (Dezaki 2007). Traditionally, GHSR couples to the Ga₄/11 signaling pathway. Therefore, mechanisms by which AG promotes to GHSR coupling to Ga₁/0 were investigated. Recent reports demonstrate that heterodimer formation with SST5 promotes GHSR coupling to Ga₁/0 in the β-cell (Park, Jiang et al. 2012).

Other reports suggest that AG inhibits insulin secretion by induction of insulinoma-associated protein (IA-2β), the β-cell autoantigen for type 1 diabetes (Doi, Shono et al. 2006). Ghrelin knockout mice have decreased levels of Ucp2 mRNA in the pancreas (Sun, Asnicar et al. 2006). UCP2 decreases insulin secretion from the β-cell, and therefore, it was suggested that AG may act through UCP2 to regulate insulin levels (Sun, Asnicar et al. 2006). In support of a UCP2 mediated mechanism, an in vitro study demonstrated that AG leads to upregulation of the AMPK–UCP2 pathway in the β-cell resulting in an inhibition of insulin secretion (Wang, Nishi et al. 2010). This pathway is independent of the IA-2β pathway (Doi, Shono et al. 2006). Taken together, many
mechanisms have been proposed whereby AG acts directly on pancreatic islets to regulate insulin secretion. A small body of evidence suggests that icv administration of AG increases plasma insulin levels in rats (Kim, Namkoong et al. 2004; Nesic, Stevanovic et al. 2008; Stevanovic, Nesic et al. 2008). However, not much focus has been placed on ghrelin’s regulation of glucose metabolism in the central nervous system (CNS).

**AG action on insulin sensitivity**

AG influences hepatic and peripheral insulin sensitivity. AG stimulates glucose production in primary hepatocytes (Gauna, Delhanty et al. 2005). Studies in hepatoma cells suggest that AG impairs insulin signaling in the liver possibly through inhibition of Akt kinase activity, as well as through upregulation of phosphoenolpyruvate carboxykinase (Murata, Okimura et al. 2002). These studies are likely mediated through a GHSR independent mechanism as GHSR expression is not found in the liver. In contrast to having a negative effect on insulin signaling in the liver, AG was shown to increase insulin-stimulated glucose uptake in adipocytes in a GHSR dependent manner (Patel, Stanley et al. 2006). A study in mice using hyperinsulinemic euglycemic clamps demonstrated that intravenous infusion of AG enhanced whole-body insulin sensitivity (Heijboer, van den Hoek et al. 2006), and hampered insulin sensitivity in the liver. Similar findings in rats demonstrated that AG inhibited Akt signaling in the liver (Barazzoni, Zanetti et al. 2007), whereas Akt signaling and expression of glucose-4 transporter (GLUT4) were enhanced in soleus muscle. These data indicate that AG enhances peripheral insulin sensitivity, and impairs insulin sensitivity in the liver,
although this is not supported by studies in humans. AG infusion during a hyperinsulinemic euglycemic clamp in humans yielded decreased basal glucose uptake and caused a reduction in insulin-stimulated glucose disposal as well as decreased glucose infusion rate (Vestergaard, Djurhuus et al. 2008). However, no effect on endogenous glucose production (EGP) was detected (Vestergaard, Djurhuus et al. 2008). These data indicate that ghrelin infusion causes peripheral insulin resistance and spares insulin sensitivity in the liver in these individuals. During this study, somatostatin was infused to reduce the confounding effects of ghrelin-induced GH secretion that is known to alter blood glucose levels and insulin sensitivity (Bratusch-Marrain, Smith et al. 1982). The authors point out that even after somatostatin infusion, GH was still significantly increased compared with controls, which could have contributed to the decreased insulin sensitivity. To determine whether the effects of AG on insulin sensitivity are independent of GH, the study was repeated in individuals with GH deficiency (Vestergaard, Gormsen et al. 2008). Ghrelin infusion in these individuals resulted in lower glucose infusion rates (GIR) and a lower rate of glucose disposal, whereas EGP was not altered by AG infusion. These data support that AG causes insulin resistance in peripheral tissues and confirms that these effects are independent of GH action. Similar findings were also reported in individuals who underwent gastrectomy that removed approximately 80% of the ghrelin-producing cells (Damjanovic, Lalic et al. 2006). An oral ghrelin mimetic taken daily for 1 year caused an increase in fasting blood glucose levels and an overall decline in insulin sensitivity (estimated by the Quicki Index method from fasting insulin and glucose) in a healthy elderly population (Nass, Pezzoli et al. 2008). Taken together, limited studies involving
AG administration in rodents demonstrate that AG seems to improve whole-body insulin sensitivity, whereas AG has a negative impact on hepatic insulin sensitivity. In humans, most data support AG acting to impair insulin sensitivity, especially in the muscle, whereas liver insulin sensitivity tends to be unaltered.

**Des-acyl ghrelin (dAG) regulation of energy metabolism**

AG has a well characterized role to promote a positive energy balance (reviewed in (Kirchner, Heppner et al. 2012)). Inconsistent reports in the literature have made it challenging to characterize the action of ghrelin’s unacylated counterpart, dAG. Original reports demonstrated that dAG did not activate GHSR or promote GH secretion (Kojima, Hosoda et al. 1999; Hosoda, Kojima et al. 2000). Therefore, researchers sought to find GHSR independent actions of dAG. In *ad libitum* fed rodents, ip injection of dAG to has no effect on feeding, whereas icv injection of dAG increases food intake through activation of orexin-expressing neurons (Toshinai, Yamaguchi et al. 2006). Other groups find that icv administration of dAG to *ad libitum* fed rodents does not alter feeding, whereas dAG ghrelin administered either centrally or peripherally to 16-h fasted rodents has an inhibitory effect on food intake (Asakawa, Inui et al. 2005). Another group replicated the inhibitory action of dAG on feeding in fasted animals and reported that this effect is mediated through activation of the corticotropin-releasing factor 2 receptor (CRF2R) (Chen, Inui et al. 2005). However, other groups find that peripherally administered dAG has no effect on food intake in either fed or fasted animals (Neary, Druce et al. 2006). One group reported that peripheral administration of dAG alone has no effect on feeding in fasted animals, but co-administration of dAG and AG reduces the
orexigenic action of AG (Inhoff, Monnikes et al. 2008) through increased activity of nesfatin-1 (a protein that has anorexigenic action) (Oh, Shimizu et al. 2006)) immunoreactive neurons. Taken together, the plethora of inconsistent results of these studies has made it difficult to confirm the effect of dAG on food intake.

Many reports demonstrate that dAG regulates adipogenesis. In rat adipocytes, dAG increases adipogenesis in a GHSR independent manner (Giovambattista, Gaillard et al. 2008). dAG increases FA uptake into 3T3-L1 adipocytes, and this effect was lost upon co-incubation with a GHSR antagonist indicating a GHSR-mediated action of dAG (Miegueu, St Pierre et al. 2011). In human visceral adipocytes, dAG promoted lipid accumulation by increasing the expression of several fat storage-related proteins, including acetyl-CoA carboxylase, fatty acid synthase, and lipoprotein lipase (Rodriguez, Gomez-Ambrosi et al. 2009). In rodents, systemic AG and dAG infusion has adipogenic action in bone marrow of rodents, whereas a GHSR specific agonist had no effect. The lack of effect of the GHSR agonist led the authors to suggest that the adipogenic of AG and dAG in bone marrow is mediated through alternative receptor (Thompson, Gill et al. 2004). Similar to AG, dAG decreases back surface temperature in rats (Inoue, Nakahara et al. 2012). In contrast to AG, the effect of dAG on back surface temperature is still apparent even in the presence of a GHSR antagonist indicating that these peptides act through different receptors to mediate body temperature. Chronic peripheral infusion of dAG has no effect on food intake or body composition in animals on a chow diet (Delhanty, Huisman et al. 2013). However, dAG prevents HFD-induced weight gain and adiposity which is independent of changes in food intake (Delhanty,
Huisman et al. 2013). Taken together, these data support that dAG regulates adipocyte metabolism. Whether this is through a GHSR independent mechanism requires further investigation.

**Des-acyl ghrelin regulation of glucose metabolism**

Despite the lack of a clearly defined receptor for dAG mediated action, several studies demonstrate that dAG has positive effects on islet cell function. Both AG and dAG prevent serum starvation-induced apoptosis in pancreatic β-cells and promote insulin secretion from these cells (Granata, Settanni et al. 2007). Most data support that dAG promotes insulin secretion both *in vitro* and *in vivo*. In INS-1E rat insulinoma cells dAG promotes insulin secretion in the presences of a GHSR antagonist (Gauna, Delhanty et al. 2006). dAG increases glucose-stimulated insulin secretion in rats (Gauna, Kiewiet et al. 2007). Furthermore, overnight intravenous infusion of dAG in healthy humans decreases blood glucose and FFA levels and increases meal-related insulin secretion (Benso, St-Pierre et al. 2012). Other studies suggest that dAG antagonizes AG action. When both AG and dAG are co-administered to rat islets, dAG abolishes the inhibitory action of AG on insulin secretion (Qader, Hakanson et al. 2008). Similarly, administration of AG alone to humans causes a reduction in insulin levels and an increase in blood glucose levels, whereas co-administration of AG and dAG has no effect on insulin or blood glucose indicating that AG inhibitory action was abolished by dAG (Broglio, Gottero et al. 2004). Additionally, intravenous infusion of AG in humans was reported to decrease insulin sensitivity, but upon co-administration of AG and dAG, insulin sensitivity was enhanced (Gauna, Meyler et al. 2004). The reason that co-
administration of both peptides causes enhanced insulin sensitivity remains elusive, but some groups support the idea that the AG:dAG ratio is a critical determinant of insulin sensitivity (Delhanty and van der Lely 2011). To support this idea, transgenic mice that express the preproghrelin gene under control of the mouse FABP4 promoter, were created to overexpress ghrelin specifically in fat tissue (Zhang, Chai et al. 2008). These mice showed elevated levels of dAG and experienced improved glucose tolerance and insulin sensitivity. However, the molecular mechanisms underlying the “AG:dAG ratio” theory needs clarification. In terms of its chronic action, 4 weeks of peripheral dAG infusion in mice on a chow diet does not alter glucose homeostasis (Delhanty, Huisman et al. 2013). However, chronic peripheral dAG infusion prevents HFD-induced glucose intolerance and insulin resistance (Delhanty, Huisman et al. 2013). Based on reports in the literature and a few pre-clinical findings demonstrating that dAG promotes insulin secretion and improves insulin sensitivity, Alizé Pharma has a program specifically dedicated to developing effective dAG analogs for the treatment of T2DM and PWS (http://www.alz-pharma.com/r-and-d-projets/unacylated-ghrelin-uag). However, a better understanding of dAG physiology, pharmacology and receptor mediated actions are required before a safe and effective therapy can be developed.

Mouse models for altered ghrelin, Ghsr and Goat function

The endogenous role of the ghrelin system in regulating energy metabolism is somewhat controversial based on loss of function rodent models. Mice lacking Ghrelin, Ghsr, or Goat expression have normal food intake and body composition when fed a chow diet (Sun, Ahmed et al. 2003; Sun, Wang et al. 2004; Sun, Butte et al. 2008;
Kirchner, Gutierrez et al. 2009). Moreover, deletion of either Ghrelin, Ghsr, or Goat in leptin deficient mice does not improve the obese phenotype (Sun, Asnicar et al. 2006; Ma, Lin et al. 2012) (Kirchner, Heppner et al., in review PLoSOne). A mild decrease in body weight and fat mass is seen in mice with a simultaneous deletion of both Ghrelin and Ghsr (Ghrelin-/-Ghsr-/- double knockout) which a result of increased energy expenditure and locomotor activity and independent of food intake (Pfluger, Kirchner et al. 2008). Interestingly, chronic exposure to HFD reveals the role of the endogenous ghrelin system. When Ghrelin or Ghsr deficient mice are placed on a HFD directly after weaning they gain less weight and body fat mass compared to wild-type (WT) controls (Wortley, del Rincon et al. 2005; Zigman, Nakano et al. 2005). The decreased body weight and fat mass in Ghrelin-/- mice is attributed to a lower respiratory quotient that alters metabolic fuel preference to favor fat oxidation (Wortley, del Rincon et al. 2005), whereas Ghsr-/- mice have a decreased body weight and fat mass as a result of decreased food intake (Zigman, Nakano et al. 2005). Similarly, Goat-/- mice fed a diet rich in medium-chain triglycerides, the acyl substrate of GOAT, gain less body weight and fat mass compared to WT controls. Collectively, the data in the literature suggest that the endogenous ghrelin system promotes peripheral lipid storage in the presence of excess calories.

Based on loss of function models, the exact role of the endogenous ghrelin system in regulating glucose metabolism is also unclear. Consistent reports have established that systemic administration of AG acts acutely to impair glucose tolerance by inhibiting insulin secretion in both rodent and humans (Dezaki, Hosoda et al. 2004; Tong, Prigeon
et al. 2010). However, inconsistent reports in the literature have made it difficult to establish the endogenous role of ghrelin in regulating glucose metabolism. Ghrelin-/- and Goat-/- mice have improved glucose tolerance and increased glucose-stimulated insulin secretion (GSIS) when fed a standard chow diet (Sun, Asnicar et al. 2006; Zhao, Liang et al. 2010). However, others have reported no differences in glucose tolerance in Ghrelin-/- mice (Pfluger, Kirchner et al. 2008) and Goat-/- mice (Kirchner, Gutierrez et al. 2009). Mice lacking Ghsr have a similar glucose tolerance, but decreased GSIS compared to WT mice (Longo, Charoenthongtrakul et al. 2008; Lin, Saha et al. 2012). However, when the animals age, Ghsr-/- mice develop improved glucose tolerance compared to aged WT controls and GSIS is similar in both groups (Lin, Saha et al. 2012). Ghsr-/- and Ghrelin-/- mice display a similar phenotype when subjected to hyperinsulinemic euglycemic clamps (Qi, Longo et al. 2011; Lin, Saha et al. 2012) (Sun, Asnicar et al. 2006). When compared to WT controls, both Ghsr-/- and Ghrelin-/- mice have increased glucose infusion rate (GIR), increased glucose disposal (Rd), and decreased endogenous glucose production (EGP) indicating improved hepatic insulin sensitivity, as well as overall enhanced peripheral insulin sensitivity. Leptin deficient (ob/ob) mice with ghrelin ablation have improved glucose tolerance compared to ob/ob mice (Sun, Asnicar et al. 2006), whereas ablation of Goat in ob/ob mice does not lead to an improvement in glucose tolerance (Kirchner, Heppner et al., in review PLoSOne). Ablation of Ghsr in ob/ob mice paradoxically worsens glucose tolerance in these animals (Ma, Lin et al. 2012). Of further controversy is the role of the ghrelin system in maintaining glucose homeostasis under a calorie restricted diet. Ghrelin-/- and Ghsr-/- mice (10 weeks old) placed on a 50% calorie restricted diet have significantly lower
blood glucose levels when compared to WT controls (Sun, Butte et al. 2008). Another group reports that Ghrelin−/− and Goat−/− mice become severely hypoglycemic to the point of death when placed on a 60% calorie restricted diet which authors attribute to the lack of AG induced GH secretion (Zhao, Liang et al. 2010; Li, Sherbet et al. 2012). The authors demonstrate that when Goat−/− mice are treated with either AG or GH, glycemic levels are restored to the level of WT mice (Zhao, Liang et al. 2010). However, the hypoglycemic phenotype is not supported by other groups (Gahete, Cordoba-Chacon et al. 2012; Yi, Heppner et al. 2012; Briggs, Lockie et al. 2013). Although GH levels significantly rise during calorie restriction, adult-onset isolated GH deficient (AOiGHD) mice maintain similar blood glucose levels as their controls during calorie restriction (Gahete, Cordoba-Chacon et al. 2012) indicating that GH is not essential to maintain glycemia during calorie restriction. Furthermore, our group used the same calorie restriction paradigm as Zhao et al., but we were not able to replicate the hypoglycemic phenotype in mice that lack Ghrelin, Ghsr or Goat even when fat mass levels reached ~0% (Yi, Heppner et al. 2012). Taken together, the use of different fasting paradigms, interactions with other circulating hormones (ie leptin) as well as different ages of genetically altered mice are all major factors that have led to inconsistent reports characterizing the endogenous role of the ghrelin system in regulating glucose metabolism.

**Ghrelin, GHSR and GOAT antagonists**

The potent orexigenic and adipogenic properties of AG made it a popular candidate for treating anorexia nervosa (AN) and chronic diseases associated with cachexia such as
cancer, chronic heart failure and chronic obstructive pulmonary disease. Promising effects on feeding and weight gain were seen in rodent models of cancer cachexia (Hanada, Toshinai et al. 2003; DeBoer, Zhu et al. 2007) as well as in clinical studies in patients suffering from cachexia (Nagaya, Moriya et al. 2004; Neary, Small et al. 2004) and AN (Hotta, Ohwada et al. 2009). However, no improvements in weight gain were reported in other human studies (Miljic, Pekic et al. 2006; Strasser, Lutz et al. 2008). In addition to treating disorders associated with a negative energy balance, ghrelin is a popular target for obesity and T2DM pharmacotherapies. Ghrelin receptor antagonists successfully reduce body weight and improve glucose homeostasis in rodents (Asakawa, Inui et al. 2003; Esler, Rudolph et al. 2007), but have not been able to successfully reduce obesity or T2DM in humans (Zhao and Liu 2006). Recently, an inhibitor for the ghrelin acylating enzyme (GOAT inhibitor) was created which has beneficial effects on energy and glucose metabolism in rodents (Barnett, Hwang et al. 2010). Mice on an medium-chain triglyceride diet chronically treated with the GOAT inhibitor have reduced body weight and fat mass gain. Furthermore, acute pretreatment with the GOAT inhibitor prior to an ip glucose challenge causes a significant increase in insulin response accompanied by a reduction in blood glucose excursions. These effects were not apparent in ghrelin knockout mice confirming that the reduction in fat mass gain and improvement in glucose tolerance is mediated by acyl ghrelin inhibition. Whether GOAT inhibitors will have similar beneficial effects in humans without adverse side effects requires further investigation. Taken together, the lack of success of ghrelin and ghrelin receptor targeted therapies for alleviating metabolic disorders in humans indicates that a further examination of ghrelin physiology and pharmacology is needed.
**Dissertation aims**

The present study examines how ghrelin action on energy and glucose metabolism is modulated by altering the acyl side-chain of the ghrelin peptide. The most abundant form of circulating ghrelin is dAG. This form of ghrelin exerts weak agonism on GHSR making its biological activity through GHSR a controversial topic in the field. The predominant form of AG found in circulation is octanoyl (C8) ghrelin. Data in the literature provide evidence that ghrelin can be acylated with FAs of varying side-chain lengths, and furthermore, these FAs can originate from the diet. The metabolic action of ghrelin acylated with different FAs is unknown. These current data demonstrate that 1) altering the fatty acid side-chain length of the ghrelin molecule modulates ghrelin action on GHSR activation, food intake, and body composition; 2) dAG induces adiposity via GHSR in the CNS; 3) C8-acylated ghrelin (AG) and dAG act in the CNS to regulate glucose-stimulated plasma insulin levels; 4) calorie restriction and age impact the endogenous GOAT-Ghrelin system’s regulation of glucose tolerance.
CHAPTER 2

Acylation type determines ghrelin's effects on energy homeostasis in rodents
Abstract

Ghrelin is a gastrointestinal polypeptide that acts through the ghrelin receptor (GHSR) to promote food intake and increase adiposity. Activation of GHSR requires the presence of a fatty-acid (FA) side-chain on amino acid residue serine 3 of the ghrelin molecule. However, little is known about the role that the type of FA used for acylation plays in the biological action of ghrelin. We therefore evaluated a series of differentially acylated peptides to determine whether alterations in length or stability of the FA side chain have an impact on the ability of ghrelin to activate GHSR in vitro or to differentially alter food intake, body weight, and body composition in vivo. Fatty acids principally available in the diet (such as palmitate C16) and therefore representing potential substrates for the ghrelin-activating enzyme ghrelin O-acyltransferase (GOAT) were used for dose-, time-, and administration/route-dependent effects of ghrelin on food intake, body weight, and body composition in rats and mice. Our data demonstrate that altering the length of the FA side-chain of ghrelin results in the differential activation of GHSR. Additionally, we found that acylation of ghrelin with a long-chain FA (C16) delays the acute central stimulation of food intake. Lastly, we found that, depending on acylation length, systemic and central chronic actions of ghrelin on adiposity can be enhanced or reduced. Together our data suggest that modification of the FA side-chain length can be a novel approach to modulate the efficacy of pharmacologically administered ghrelin.
Introduction

Ghrelin is a 28-amino acid peptide secreted mainly from the X/A-like cells of the stomach (Kojima, Hosoda et al. 1999; Date, Kojima et al. 2000; Ariyasu, Takaya et al. 2001). A unique feature of ghrelin is the esterification with an 8-\(n\) acyl group at the serine in position 3. Acylation of ghrelin occurs during post-translational modification in which an \(n\)-octanoic acid is esterified to the serine 3 residue of the peptide (Gutierrez, Solenberg et al. 2008; Yang, Brown et al. 2008). The enzyme that catalyzes this reaction is ghrelin O-acyltransferase (GOAT), which is a membrane-bound protein highly expressed in the stomach (Gutierrez, Solenberg et al. 2008; Yang, Brown et al. 2008). Ghrelin is found in circulation in both the acyl and des-acyl forms (Kojima, Hosoda et al. 1999; Hosoda, Kojima et al. 2003). However, the presence of the fatty acid (FA) side chain is required for the binding of ghrelin and activation of its G protein-coupled receptor known as the GH-secretagogue receptor (GHSR) (Kojima, Hosoda et al. 1999). This receptor retains significant baseline constitutive activity (Holst, Cygankiewicz et al. 2003; Holst, Holliday et al. 2004; Petersen, Woldbye et al. 2009) and is highly expressed in the hypothalamus (Howard, Feighner et al. 1996; Zigman, Jones et al. 2006), which is the nexus for the regulation of energy balance. The agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons of the hypothalamus express GHSR (Nogueiras, Tschop et al. 2008; Castaneda, Tong et al. 2010) and are a primary target of ghrelin (Nakazato, Murakami et al. 2001; Wang, Saint-Pierre et al. 2002; Cowley, Smith et al. 2003; Briggs and Andrews 2011). Ghrelin stimulation of these neurons (Willesen, Kristensen et al. 1999) results in an increase in NPY/AgRP peptide release in the paraventricular nucleus (PVN). NPY activates Y1 and Y5 (Fekete, Sarkar
et al. 2002) receptors, whereas AgRP antagonizes melanocortin 4 receptor in the PVN (Cone, Lu et al. 1996). Additionally, ghrelin activation of GHSR on NPY/AgRP neurons promotes the release of γ-aminobutyric acid (Cowley, Smith et al. 2003), which acts to inhibit the anorectic effect of proopiomelanocortin-expressing neurons in the arcuate nucleus. The collective ghrelin-induced activation of GHSR on NPY/AgRP-expressing neurons in the hypothalamus is increased food intake (Wren, Small et al. 2000; Nakazato, Murakami et al. 2001; Wren, Seal et al. 2001; Perez-Tilve, Heppner et al. 2011) and adiposity (Tschop, Smiley et al. 2000; van der Lely, Tschop et al. 2004).

Although the acylated form of ghrelin that was initially identified was octanoyl ghrelin (Kojima, Hosoda et al. 1999), GOAT can actually acylate ghrelin with FA of different lengths (Gutierrez, Solenberg et al. 2008; Yang, Brown et al. 2008; Ohgusu, Shirouzu et al. 2009). Furthermore, these FA can have dietary origin since mice were found to have circulating ghrelin acylated with a C7-FA side chain after being fed a diet rich in nonendogenously produced C7-chain FA (Nishi, Hiejima et al. 2005; Kirchner, Gutierrez et al. 2009). In addition, ghrelin esterified by n-decanoic acid was isolated from human stomach and plasma (Hosoda, Kojima et al. 2003). This form of ghrelin has the same potency to increase [Ca²⁺] in GHSR-expressing cells and to stimulate GH release in anesthetized rats as octanoyl ghrelin (Hosoda, Kojima et al. 2003). One report demonstrated that altering the structure of the fatty acid side chain alters ghrelin's ability to activate GHSR in human embryonic kidney-293 (HEK-293) cells (Bednarek, Feighner et al. 2000), and another group found that replacing the ester bond linking the octanoic acid to the Ser3 residue of the ghrelin peptide with a more chemically stable
ether or thioether bond resulted in similar GHSR activation in Chinese hamster ovary-
GHSR62 cells as native ghrelin (Matsumoto, Hosoda et al. 2001). Interestingly,
administering ghrelin stabilized with a thioether bond iv to rats delayed the onset of GH
secretion, although total GH secretion of the stable vs. native ghrelin was similar.
Despite these findings exploring GHSR activation by ghrelin isoforms in cell-based
assays and on GH secretion, it is unclear whether these effects will translate to the in
vivo action of ghrelin on energy metabolism.

Circulating ghrelin levels change in pathophysiological conditions involving perturbed
energy balance, such as obesity (Tschop, Weyer et al. 2001; English, Ghaetei et al.
2002; Hansen, Dall et al. 2002; Shiiya, Nakazato et al. 2002; le Roux, Patterson et al.
2005) and diabetes (Poykko, Kellokoski et al. 2003), and there are inconsistent reports
that surgical procedures intended to reduce obesity and diabetes, such as Roux-en-Y
gastric bypass, also result in changes in plasma ghrelin levels (Cummings, Weigle et al.
2002; Pournaras and le Roux 2010). However, it is unknown whether different metabolic
conditions have an impact on the levels of different acyl ghrelin isoforms, and the
accurate measurement of different acyl ghrelin levels is still unfeasible due to the lack of
specific high-throughput assays. Taken together, the changes in ghrelin associated with
the metabolic disease, and the endogenous existence of ghrelin acylated with different
fatty acids, warrants further investigation of the possible alternative physiological actions
of these isoforms.

The aim of this study was to systematically dissect the in vitro and in vivo actions of
synthetic ghrelin compounds acylated with FA of variable length (C2-C16) and stability
to determine whether these alterations affect the action of ghrelin on GHSR activation, food intake, body weight, and body composition.

**Materials and Methods**

*Peptide synthesis*

Rat ghrelin was synthesized using *in situ* neutralization for Boc chemistry, purified by preparative chromatography, and characterized by HPLC and mass spectral analysis, as described previously (Tschop, Smiley et al. 2000). The native ghrelin used for these experiments contained an octanoic acid (C8) forming an ester bond at the Ser3 hydroxyl side chain. Ghrelin isoforms were synthesized using the 28-amino acid sequence of rat ghrelin, but the number of carbons in the FA side chain was altered (C2-C16). For the stabilized analog, the ester bond at the Ser3 was replaced with an isostere that has an amino group on the side chain. The amino group was acylated with octanoic acid to form an amide bond, which is less prone to hydrolysis, resulting in enhanced stability of bond of the octanoyl group to the peptide molecule. These peptide acyl amides are denoted as SC8 and SC16 to signify the absence of the labile acyl ester of endogenous ghrelin isoforms.

*Transfections and tissue culture*

COS-7 cells were grown in DMEM 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. The cells were transfected using the calcium phosphate precipitation method with chloroquine addition as previously described (Holst, Zoffmann et al. 1998).
Phosphatidylinositol turnover

One day after transfection, COS-7 cells were incubated for 24 h with 5 μCi of [3H]-myo-inositol (Amersham Bioscience, Piscataway, NJ; PT6-271) in 1 ml medium supplemented with 10% fetal calf serum, 2 mM glutamine, 180 U/ml penicillin, and 45 μg/ml streptomycin. Cells were washed twice in buffer, 20 mM HEPES (pH 7.4), supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 10 mM glucose, and 0.05% (wt/vol) bovine serum and were incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37 C for 30 min. After stimulation with various concentrations of peptide for 45 min at 37 C, cells were extracted with 10% ice-cold perchloric acid followed by incubation on ice for 30 min. The resulting supernatants were neutralized with KOH in HEPES buffer, and the generated [3H]-inositol phosphate was purified on Bio-Rad Laboratories (Hercules, CA) AG 1-X8 anion-exchange resin as described. Determinations were made in duplicate.

Animals

Male C57BL/6 mice (8 wk old; Jackson Labs, Bar Harbor, ME) and male Long-Evans rats (300–350 g; Harlan, Indianapolis, IN) were maintained on a standard chow diet (Teklad; Harlan). After receiving intracerebroventricular (icv) surgery, animals were singly housed on a 12-h light, 12-h dark cycle at 22 C with free access to food and water unless noted otherwise. Mice that received C6 or C16 acylated ghrelin were housed in an indirect calorimetry system (TSE LabMaster, Bad Homburg v.d.H., Germany) for the study period. Mice infused with C2, C12, or C14 were housed in
conventional cages. Each study had a saline and C8 control group that was housed in the same manner as their corresponding treatment groups. Animals that received chronic sc injections were group housed (four mice per cage) in conventional cages. Ghsr-/-. mice (8–12 wk) were received from Regeneron Pharmaceuticals (Tarrytown, NY) (Wortley, Anderson et al. 2004; Abizaid, Liu et al. 2006; Pfluger, Kirchner et al. 2008) and bred in our facilities as described previously (Pfluger, Kirchner et al. 2008). All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

**Acute icv cannula implantation in rats**

A 22-gauge stainless steel cannula (Plastics One, Roanoke, VA) was stereotaxically implanted into the third ventricle of rats according to the following coordinates: −2.2 mm posterior to bregma and to a depth of −7.5 mm from the surface of the brain, with bregma and lambda being horizontal. Dental acrylic secured the cannula to the skull and an obturator extending 1 mm below the guide cannula was inserted. After a week of recovery, correct placement of the cannula was verified by icv administration of angiotensin II (1 μg/μl of 0.9% saline). Rats that failed to drink a minimum of 5 ml of water within 30 min were removed from the studies.

**Determination of the acute effect of icv ghrelin on food intake**

Food was weighed before icv injections which occurred at the beginning of the light phase. Briefly, an internal cannula (Plastics One) was connected to polyethylene-50 tubing, and the tubing was connected to a 25-μl Hamilton syringe (Hamilton, Reno, NV).
The peptide was drawn into the tubing, the internal cannula was inserted, and a 1-μl volume of the peptide or saline was infused. Food intake was recorded 2, 4, and 24 h after injection. Doses were used as indicated and they included 0.3, 0.9, and 3 nmol (equivalent to 1, 3, and 10 μg of ghrelin isoforms).

*Intracerebroventricular infusions in mice*

Ghrelin was dissolved in isotonic saline and infused icv at a dose of 5 nmol/d per mouse (5 nmol = 15 μg of ghrelin isoforms) using osmotic minipumps (1007D; Alzet, Cupertino, CA), prepared following the manufacturer's instructions. Male C57BL/6 mice were anesthetized using 5% isoflurane in oxygen in an induction chamber and then maintained on 2.5% isoflurane delivered by a nose cone. Mice were stereotaxically implanted (David Kopf Instruments, Tujunga, CA) with a cannula (brain infusion kit no. 3; Alzet) placed in the lateral cerebral ventricle using the coordinates −0.7 mm posterior to bregma, −1.2 mm lateral to the midsagittal suture, and to a depth of −2.5 mm from the surface of the brain, with bregma and lambda being horizontal. A polyethylene catheter attached the cannula to an osmotic minipump that was sc implanted in the interscapular region. The pumps infused either ghrelin or saline at a rate of 0.5 μl/h for 7 d. After surgery, the animals received a single sc dose of 0.28 mg/kg buprenorphin (Buprenex; Reckitt Benckiser Healthcare, Richmond, VA) and were maintained on a heated surface until full recovery. Food intake and body weight were measured daily. Body composition was analyzed immediately before surgery and again on the final day of infusion.
Body composition measurements

Whole-body composition (fat and lean mass) was measured using nuclear magnetic resonance technology (Tinsley, Taicher et al. 2004) (EchoMRI-100; Echo Medical Systems, Houston, TX).

Subcutaneous injections in mice

Mice were given a daily sc injection of saline, C8, C16, SC8, or SC16-acylated ghrelin. Ghrelin compounds were dissolved in saline and administered at a dose of 60 nmol/d per mouse (60 nmol = 200 μg of ghrelin isoforms). Food intake and body weight were measured daily. Body composition was measured before the initial injection and again after the final injection.

Calculations and statistical analysis

For inositol phosphate (IP) accumulation analysis in COS-7 cells, EC50 values were determined by nonlinear regression using Prism version 4.0 software (GraphPad Software, San Diego, CA). Statistical analysis for animal studies was performed using GraphPad Prism version 5.0 (GraphPad Software). The differences among treatments were assessed by using a one-way ANOVA and a Tukey's post hoc test. For analysis of time-dependent differences in body weight and food intake, two-way ANOVA were used with the Bonferroni post hoc. All results are given as means ± SEM. Results were considered statistically significant when $P < 0.05$. 
Results

Altering the fatty acid side-chain length of ghrelin results in variable activation of GHSR in COS-7 cells

We assessed synthetic ghrelin compounds acylated with different side-chain lengths (C2-C16) for their ability to activate GHSR by determining dose-response curves of phosphatidylinositol turnover (Figure 2.1). The receptor activation of the isoforms was compared with that of native C8-acylated ghrelin induced receptor activation. Ghrelin acylated with a C8-FA stimulated IP turnover with a potency (EC50 = 0.43 nM) similar to what has previously been reported (Holst, Cygankiewicz et al. 2003; Holst, Brandt et al. 2005). C2-acylated ghrelin did not stimulate IP accumulation in these cells (Figure 2.1A), indicating that a FA side chain greater than 2 carbons is necessary to induce GHSR activation. Ghrelin acylated with a C6 medium chain FA (Figure 2.1B) induced similar IP accumulation as C8-acylated ghrelin. Ghrelin acylated with longer FA side chains (C12 and C14, Figure 2.1, C and D) shifted the dose response curves to the right with 80- and 37-fold decrease in potencies, respectively, compared with native C8-acylated ghrelin. Unlike ghrelin acylated with long-chain fatty acids of C12 and C14, ghrelin acylated with a C16 FA (Figure. 2.1E) caused a similar induction of IP accumulation as C8-acylated ghrelin.

Effect of acute icv injection of the different acyl ghrelin isoforms on food intake in rats

To determine whether altering the length of the FA side chain of ghrelin influences acute food intake, we conducted a preliminary screening of C2-, C6-, C8-, C12-, C14-, and
C16-acylated ghrelin by administering these compounds icv (0.9 nmol) to rats at the beginning of the light phase (Figure 2.2). Comparable with previous reports (Wren, Small et al. 2000; Nakazato, Murakami et al. 2001; Perez-Tilve, Heppner et al. 2011), rats treated with C8-acylated ghrelin had increased food intake at 2 and 4 h after injection compared with saline-treated controls ($P < 0.05$, C8 vs. saline, Student's $t$ test). Ghrelin acylated with C2, C6, C12, C14, and C16 FA had no effect on food intake 2 and 4 h after injection compared with saline controls. Unexpectedly, rats treated with C16-acylated ghrelin, which had normal food intake at 2 and 4 h, had a significant increase in food intake after 24 h compared with saline controls ($P < 0.05$, C16 vs. saline, Student's $t$ test), whereas food intake in the C2-, C6-, C8-, and C14-treated groups was similar compared with saline controls. No change in body weight was noted 24 h after injection in any group (data not shown). Taken together, these data suggest that modification of ghrelin with a C16 FA delays the induction of food intake in response to centrally administered ghrelin.

*Effect of chronic icv infusion of different acyl-ghrelin isoforms on food intake, body weight, and adiposity in mice*

To assess the chronic central effects of C2-, C6-, C8-, C12-, C14-, or C16-acylated ghrelin, mice were infused with each of the ghrelin analogs (5 nmol/d per mouse) via a sc implanted minipump that was attached to a cannula placed in the lateral ventricle of the brain. The effects on food intake and body weight are summarized in Table 2.1. Cumulative food intake was increased in the C12-acylated ghrelin group compared with saline-treated controls after 6 d of infusion (Table 2.1). Body weight was increased 6 d
after treatment in the C8-, C12-, and C14-treated mice. Animals administered with C2-acylated ghrelin had no change in fat mass after the infusion period (Figure 2.3A), whereas mice receiving C6-, C8-, C12-, C14-, and C16-acylated ghrelin all had increased fat mass (Figure 2.3, B–E) compared with saline-treated controls. Although animals treated with C14-acylated ghrelin experienced an increase in fat mass, this was significantly less than animals infused with C8-acylated ghrelin. No change in lean mass was observed in any group (data not shown). Mice infused with C6- and C16-acylated ghrelin were placed in an indirect calorimetry system for the duration of the study for further analysis. There were no differences in energy expenditure, respiratory quotient, or locomotor activity compared with C8-acylated ghrelin (data not shown).

The lack of effect on fat mass observed in the C2-treated group as well as the decreased effect of C14-acylated ghrelin compared with C8-treated animals is consistent with our GHSR activation assay in which we found that C2-acylated ghrelin lacked the ability to induce GHSR activation and that C14-acylated ghrelin had decreased potency compared with C8-acylated ghrelin. Interestingly, we found that C12-acylated ghrelin had a similar potency to increase fat mass as C8-acylated ghrelin; however, C12-acylated ghrelin had a less potent effect on IP accumulation compared with C8-acylated ghrelin (Figure 2.1C). A possible explanation for this is that C12-acylated ghrelin may have GHSR-independent actions.
C12-acylated ghrelin requires GHSR to increase food intake and fat mass in mice

To explore the possibility that C12-acylated ghrelin has GHSR-independent action, we infused Ghsr-/- mice icv with C8- or C12-acylated ghrelin (5 nmol/d per mouse). Neither C8-nor C12-acylated ghrelin had an effect on food intake (Figure 2.4A) during the infusion period. We also found that body weight (Figure 2.4B), fat mass (Figure 2.4C), and lean mass (data not shown) were not altered in either of the ghrelin treatment groups after the 7-d central treatment. From these data we concluded that C12-acylated ghrelin requires GHSR to mediate its effects in mice.

Effect of icv C16-acylated ghrelin on acute feeding in rats

To confirm the delayed effect of C16-acylated ghrelin on acute feeding, we administered doses of 0.3, 0.9, and 3 nmol of C16- and C8-acylated ghrelin and compared them with saline-treated controls (Figure 2.5). Consistent with our findings in Figure 2.2 as well as previous reports (Wren, Small et al. 2000; Nakazato, Murakami et al. 2001; Perez-Tilve, Heppner et al. 2011), icv C8-acylated ghrelin induced a significant increase in food intake at early time points (2 and 4 h) with doses of 0.9 and 3 nmol, whereas C16-acylated ghrelin had no effect at these time points (Figure 2.5, A and B). C8-acylated ghrelin had no effect on 24-h food intake, whereas doses of 0.9 and 3 nmol of C16-acylated ghrelin significantly increased feeding at 24 h compared with saline-treated controls (Figure 2.5C). The reason that C16-acylated ghrelin has a delayed effect on food intake is not clear, but a possible explanation is due to increased stability of the FA side chain to the ghrelin peptide or enhanced association with plasma proteins, such as albumin.
Effect of chronic peripheral administration of C16-acylated ghrelin and stabilized ghrelin compounds in mice

Ghrelin receptor activity is dependent on Ser3 acylation, and the ester bond is highly prone to hydrolysis by circulating esterases, rendering the duration of active ghrelin action relatively short (De Vriese, Gregoire et al. 2004). Thus, stabilizing this ester bond should increase the duration of action and enhance the efficacy of ghrelin. We hypothesized that one explanation for the delayed action of C16-acylated ghrelin on acute food intake is due to enhanced resistance to enzymatic cleavage of the ester bond. If so, chronic peripheral treatment with C16-acylated ghrelin or C8-acylated ghrelin with a stabilized bond (SC8) between the FA and ghrelin peptide should prolong the action of ghrelin and enable it to have a more potent effect on food intake, body weight, and adiposity. We therefore treated mice with daily sc injections of C8, C16, SC8, or SC16 ghrelin (Figure 2.6). Mice were housed four animals per group (n = 2 cages per treatment). Food intake was similar among groups but tended to be increased in the cages that received the C16 compared with saline-treated controls after 6 d of injections (cumulative average per mouse: saline, 24.07 ± 2.41 g; C8-acylated ghrelin, 27.40 ± 0.12 g; SC8-acylated ghrelin, 27.23 ± 1.73 g; C16-acylated ghrelin, 33.34 ± 1.21 g; SC16-acylated ghrelin, 28.90 ± 0.38 g). Body weight was significantly increased in all ghrelin treatment groups compared with saline-treated controls (Figure 2.6A). Body weight was significantly increased in the C16- and SC16-acylated ghrelin treatment groups relative to the C8 and SC8 treatment groups. Consistent with the effects on body weight, all ghrelin treatment groups experienced a significant increase
in fat mass (Figure 1.6B). Again, both C16-acylated compounds more effectively increased fat mass compared with both C8-acylated compounds (Figure 2.6B). No change in lean mass was observed in any of the treatment groups (data not shown). These data suggest that acylation of ghrelin with a C16 FA enhances the efficacy of pharmacological doses of ghrelin.

Discussion

Previous reports using cell-based assays demonstrate that altering the fatty acid side-chain length of the ghrelin molecule alters its ability to bind and activate GHSR (Bednarek, Feighner et al. 2000). The ability of the enzyme GOAT to use dietary lipids as a substrate to acylate ghrelin, coupled with studies documenting the existence of ghrelin acylated with different fatty acids, led us to investigate whether altering the fatty-acid side-chain length of the ghrelin molecule will cause the peptide to have differential biological action on food intake, body weight, and/or adiposity. In addition to finding differential GHSR activation in cell-based assays, we report novel findings on energy metabolism of ghrelin acylated with FA of varying length (C2-C16) and chemical stabilities in rodents. Specifically, the presence of a FA side chain longer than C2 is required for ghrelin to activate the GHSR receptor and to exert its biological effects on food intake, body weight, and adiposity. Our chronic studies are in accordance with previous findings that demonstrate ghrelin’s effects on adiposity can be independent of changes in food intake (Tschop, Smiley et al. 2000; Theander-Carrillo, Wiedmer et al. 2006; Perez-Tilve, Heppner et al. 2011; Pfluger, Castaneda et al. 2011) and body weight gain (Theander-Carrillo, Wiedmer et al. 2006). As a rule, we find that GHSR
activation in cells provides a reliable prediction of how ghrelin analogs perform on *in vivo* adiposity, but interestingly, this is not always the case. Ghrelin acylated with a C12 or a C14 FA exhibits a decreased potency to activate GHSR when compared with native C8-acylated ghrelin. As expected, chronic central treatment of C14-acylated ghrelin to mice has a weak effect on increasing adiposity. Unexpectedly, C12-acylated ghrelin increases adiposity in mice with a similar potency as C8-acylated ghrelin. We hypothesized that this could be a result of a GHSR-independent action of C12-acylated ghrelin. To test this, we icv infused C12-acylated ghrelin to *Ghsr/-/-* mice and found that C12-acylated ghrelin loses its effects on adiposity in *Ghsr/-/-* mice. Thus, GHSR is essential for the action of C12-acylated ghrelin.

These studies highlight the complexity of the *in vivo* ghrelin system. Many reports demonstrate that GHSR is able to heterodimerize with other G protein-coupled receptors (Rediger, Piechowski et al. 2011; Kern, Albarran-Zeckler et al. 2012). Acylation of ghrelin with a C12 rather than a C8 FA may differentially affect the heterodimerization of these receptors. This could explain the strong effect of C12-acylated ghrelin on adiposity in rodents that we found as well as weak GHSR activation in COS-7 cells that are not transfected with these additional G protein-coupled receptor. Our data suggest that a combination of both *in vitro* screening and *in vivo* testing of drug candidates may be an important determinant for the development of effective therapies based on targeting GHSR activity.
The most striking findings of the present report are the acute and chronic actions of C16-acylated ghrelin in rodents. Despite having a similar activation of GHSR in COS-7 cells, centrally administered C16-acylated ghrelin in rats reveals a unique delayed time course of action on food intake. Comparable prolonged effects can be observed in many pharmacological agents that are commonly acylated with a C16 FA. We explored the possibility that the prolonged onset of increased food intake could be due to the increased stability of the C16 FA to the ghrelin peptide and therefore might increase the efficacy of chronic peripheral ghrelin administration. We found that chronic peripheral treatment with ghrelin acylated with a C16 FA side chain had a greater ability to increase body weight and fat mass than ghrelin acylated with a C8-FA side chain.

We tested a stabilized form of both C8- and C16-acylated ghrelin, in which the ester bond linking the FA to the Ser3 residue was replaced with a nonhydrolysable amide bond. The stabilized ghrelin compounds did not increase the efficacy of ghrelin on body weight or fat mass compared with their nonstabilized counterparts. However, acylation of ghrelin with a C16 FA, regardless of whether the ester bond is stabilized, enhances ghrelin's effects on body weight and adiposity. This demonstrates that C16 acylation increases the potency of ghrelin through a mechanism other than enhanced stability of the FA to the peptide molecule.

The mechanisms responsible for the enhancement of C16-acylated ghrelin on energy metabolism require further clarification. It has been reported that the octonyl side chain of ghrelin provides an anchor to bind the peptide to the cell membrane (Grossauer,
It is possible that the C16-FA side chain acts as a more effective anchor and therefore has a decreased amount of clearance from the circulation than C8-acylated ghrelin. In a related fashion, it is known that serum albumin binds long-chain FAs (Hamilton, Era et al. 1991) and acylated peptides, such as liraglutide and detemir. Consequently, it is possible that the C16 FA ghrelin binds to albumin, allowing the peptide to remain in circulation for an extended period of time. This might also account for the diminished early action in acute central administration of the C16-acylated ghrelin because albumin binding could suppress such activity. The rates of clearance of C8- vs. C16-acylated ghrelin have yet to be determined, and whether a decreased rate of clearance contributes to the increased potency of chronic treatment with C16-acylated ghrelin requires further investigation. In addition to examining the rates of clearance, future studies investigating intracellular signaling pathways could illuminate possible mechanisms mediating the delayed effect on acute food intake found with icv administration of C16-acylated ghrelin. For example, AMP-activated protein kinase (AMPK) has previously been suggested to act as an intracellular energy sensor, and pharmacological activation of hypothalamic AMPK has been shown to increases food intake (Andersson, Filipsson et al. 2004). Interestingly, the orexigenic action of icv C8-acylated ghrelin administration correlates with a time-dependent increase in AMPK phosphorylation that peaks acutely at 2 h and returns to normal levels at 6 h after treatment (Lopez, Lage et al. 2008). It is possible that a delay in AMPK phosphorylation after icv administration of C16-acylated ghrelin mediates the delayed effect on food intake reported here. However, whether ghrelin’s orexigenic action can exclusively be attributed to activation of AMPK remains to be determined. Additionally, central C8-
acylated ghrelin administration causes neuronal activation in hypothalamic nuclei including the arcuate nucleus and PVN (Lawrence, Snape et al. 2002). Follow-up studies investigating induction of c-fos as a marker of neuronal activation in these hypothalamic nuclei after icv administration of the various ghrelin isoforms are underway to help clarify whether the differential effects on acute food intake are a result of alterations in neuronal activation.

Future studies examining the endogenous existence of these ghrelin isoforms and whether these isoforms are altered in metabolic diseases including obesity, cancer cachexia, and anorexia nervosa could help us understand the pathogenesis of these disorders. A study in which mice were fed diets enriched in FA of various lengths used mass spectroscopy to demonstrate that stomach-derived ghrelin can be acylated with FA of different lengths including C6, C10, and C10:1 (Nishi, Hiejima et al. 2005). Furthermore, this study demonstrated that these various ghrelin isoforms can be secreted into the circulation. Although it seems that stomach derived ghrelin has a preference for medium chain FA, it would be of interest to explore whether other tissues expressing both ghrelin and GOAT (e.g. brain, kidney, adipose tissue, small intestine, and large intestine) are able to synthesize additional ghrelin isoforms when animals are exposed to diets enriched in short- or long-chain FA.

Collectively this report provides a novel investigation of the comparison of *in vitro* vs. *in vivo* effects of synthetic ghrelin peptides of varied acyl-character. Our data demonstrate that acyl chains of differing length possess unique pharmacology and imply the
existence of differential biological action profiles for naturally occurring acyl-ghrelin isoforms. These effects are specific to GHSR activation but may involve other receptor signaling systems as well. Importantly, our data demonstrate that C16-acylation is a novel approach to enhance and prolong the efficacy of pharmacologically administered ghrelin.

Based on these data, it is clear that the in vivo action of various ghrelin isoforms can not always be predicted from cell-based GHSR activation assays. Furthermore, a multitude of recent reports indicate that dAG has biological action (Benso, St-Pierre et al. 2012; Delhanty, Huisman et al. 2013; Porporato, Filigheddu et al. 2013). Therefore, our next aim was to systematically examine the chronic peripheral and central GHSR-dependent actions of dAG in comparison with C8-acylated ghrelin (AG) on food intake, body weight, body composition and glucose tolerance.
Figure 2.1: *In vitro* receptor activation in COS-7 cells transiently expressing GHSR. Specificity of the receptor activation was established by the level of IP accumulation induced by C8-acylated ghrelin. (A) In similar conditions, C2-acylated ghrelin has negligible activity. C6 and C16 have similar potency to activate GHSR as that of C8-acylated ghrelin (B and E), whereas C12 and C14 have a decreased potency (C and D).
Figure 2.2: Preliminary evaluation of ghrelin isoforms on acute food intake in rats. Rats were administered icv saline or 0.9 nmol of C2-, C6-, C8-, C12-, C14-, or C16-acylated ghrelin at the beginning of the light phase. Food intake 2 and 4 h after injection was increased in animals treated with C8-acylated ghrelin compared with saline controls. *, $P < 0.05$, C8 vs. saline, Student's $t$ test. Food intake 24 h after the injection was significantly increased in rats receiving C16-acylated ghrelin compared with saline-treated rats. *, $P < 0.05$, C16 vs. saline, Student's $t$-test ($n = 9–10$ rats per group).
### Table 1. Chronic central effects of ghrelin isoforms on food intake and body weight in mice

<table>
<thead>
<tr>
<th></th>
<th>Cumulative FI (g)</th>
<th>ΔBody weight (g)</th>
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<tbody>
<tr>
<td><strong>C2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>31.49 ± 1.24</td>
<td>1.29 ± 0.27</td>
</tr>
<tr>
<td>C8</td>
<td>35.57 ± 1.72</td>
<td>2.75 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C2</td>
<td>31.78 ± 1.60</td>
<td>1.70 ± 0.28</td>
</tr>
<tr>
<td><strong>C6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>22.72 ± 1.22</td>
<td>0.09 ± 0.42</td>
</tr>
<tr>
<td>C8</td>
<td>25.04 ± 1.26</td>
<td>1.06 ± 0.31</td>
</tr>
<tr>
<td>C6</td>
<td>23.46 ± 1.35</td>
<td>0.64 ± 0.5</td>
</tr>
<tr>
<td><strong>C12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>31.49 ± 1.24</td>
<td>1.29 ± 0.27</td>
</tr>
<tr>
<td>C8</td>
<td>35.57 ± 1.72</td>
<td>2.75 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C12</td>
<td>36.91 ± 0.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.85 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>C14</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>26.98 ± 1.0</td>
<td>0.24 ± 0.155</td>
</tr>
<tr>
<td>C8</td>
<td>30.71 ± 1.65</td>
<td>1.79 ± 0.3275&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C14</td>
<td>27.69 ± 1.36</td>
<td>1.32 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>C16</strong></td>
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<tr>
<td>Saline</td>
<td>22.72 ± 1.22</td>
<td>0.09 ± 0.42</td>
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<tr>
<td>C8</td>
<td>25.04 ± 1.26</td>
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<tr>
<td>C16</td>
<td>22.88 ± 1.85</td>
<td>0.87 ± 0.32</td>
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</tbody>
</table>

**Table 2.1:** Chronic central effects of ghrelin isoforms on food intake and body weight in mice. Cumulative food intake and change in body weight after 6 d of chronic icv infusion of C2-, C6-, C8-, C12-, C14-, or C16-acylated ghrelin (5 nmol/d per mouse) in C57/BL6 mice. a: $P < 0.05$ vs. saline (one-way ANOVA; n = 8–10 mice per group). b: $P < 0.001$ vs. saline (one-way ANOVA; n = 8–10 mice per group).
Figure 2.3: Chronic central effects of ghrelin isoforms on adiposity. C57/BL6 mice were implanted with icv minipumps that infused C2-, C6-, C8-, C12-, C14-, or C16-acylated ghrelin (5 nmol/d per mouse). C2-acylated ghrelin did not have an effect on fat mass after a 7-d infusion period (A), whereas C6- (B), C12- (C), C41- (D), and C16-acylated ghrelin (E) all increased fat mass compared with saline treated controls. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ vs. saline, one-way ANOVA. D, C14-acylated ghrelin was significantly less effective at increasing fat mass as C8-treated animal. #, $P < 0.05$ C14 vs. C8 (one-way ANOVA with Tukey post hoc; n = 8–10 animals per group). Sal, Saline.
Figure 2.4: Effects of C12-acylated ghrelin on food intake, body weight, and body composition in Ghsr-/− mice. Ghsr-/− mice were infused icv for 7 d with C8- or C12-acylated ghrelin (5 nmol/d per mouse). (A) Cumulative food intake in mice treated with C8- or C12-acylated ghrelin was similar to that of saline-treated controls 7 d after treatment. (B) Change in body weight on d 7 of treatment was similar in both experimental groups compared with saline-treated controls. (C) Change in fat mass on d 7 of treatment was also similar among all groups (n = 8 animals per group).
Figure 2.5: Central administration of C16-acylated ghrelin delays the onset of acute food intake in rats. Rats received icv saline or C8- or C16-acylated ghrelin at doses of 0.3, 0.9, and 3 nmol. A, At 2 h after the injection, rats treated with C8-acylated ghrelin experienced an increase in food intake with doses of 0.9 and 3 nmol. **, \( P < 0.01 \) C8 vs. saline (two-way ANOVA). B, At 4 h after the injection, rats treated with C8-acylated ghrelin experienced an increase in cumulative food intake with doses of 0.9 and 3 nmol. *, \( P < 0.05 \), **, \( P < 0.01 \) C8 vs. saline (two-way ANOVA). C, Rats receiving C16-acylated ghrelin had a delayed effect on food intake, and cumulative 24 h food intake was increased in rats treated with 0.9 and 3 nmol. *, \( P < 0.05 \), ***, \( P < 0.001 \) C16 vs. saline (two-way ANOVA; \( n = 9–15 \) animals per group).
Figure 2.6: Effect of chronic peripheral treatment of C16-acylated ghrelin and stabilized ghrelin compounds in mice. C57/BL6 mice were given daily sc injections of native C8-acylated ghrelin (C8), C16-acylated ghrelin (C16), stabilized C8-acylated ghrelin (SC8), or stabilized C16-acylated ghrelin (SC16) at a dose of 60 nmol/d per mouse. (A) All ghrelin-treated mice had a significant increase in body weight compared with saline-treated animals. $, P < 0.05$ vs. saline (two-way ANOVA). Both C16- and SC16-treated groups had a greater increase in body weight compared with C8. $#, P < 0.05$ C16- and SC16- vs. C8 (two-way ANOVA) and SC8-treated animals. $$, $P < 0.05$ C16 and SC16 vs. SC8 (two-way ANOVA). (B) All ghrelin-treated mice had increased fat mass after 6 d of injections. **, $P < 0.01$, ***, $P < 0.001$ vs. saline (one-way ANOVA). Mice treated with C16 and SC16 had a greater increase of fat mass compared with C8 and SC8. $#, P < 0.05$, $$, P < 0.01$, ####, $P < 0.001$ C16 or SC16 vs. C8 or SC18 (one-way ANOVA with Tukey post hoc test; n = 8 mice per group).
CHAPTER 3

Both acyl and des-acyl ghrelin act on CNS ghrelin receptors to regulate adiposity and glucose metabolism in mice
Abstract

The ghrelin receptor (GHSR) in the central nervous system (CNS) mediates hyperphagia and adiposity induced by its endogenous ligand, acyl-ghrelin (AG). There is evidence that des-acyl ghrelin (dAG) has biological activity, but this has been attributed exclusively to GHSR independent mechanisms. We combined both in vitro and in vivo approaches to test possible GHSR mediated biological activity of dAG. As expected, AG (1 µM) significantly increased IP₃ formation in HEK-293 cells transfected with human GHSR. Interestingly, after GHSR overexpression dAG (1 µM) also significantly increased IP₃ accumulation in GHSR-transfected cells, although at a maximal stimulation that was lower than AG. To determine whether dAG activates the GHSR in vivo, we gave intracerebroventricular (icv) infusion of AG and dAG to mice. AG increased feeding and adiposity in a dose-dependent manner, whereas equimolar doses of icv-dAG failed to stimulate feeding but led to a dose-dependent increase in adiposity. The highest icv administered dose (5 nmol/mouse/day) of AG and dAG increased fat mass (FM) to a similar magnitude. Chronic icv infusion of AG or dAG (5 nmol/mouse/day) induced hyperinsulinemia during a glucose tolerance test. In contrast to AG, dAG failed to increase FM or plasma insulin when administered subcutaneously at doses that were efficacious when delivered icv. During a hyperinsulinemic-euglycemic clamp, icv-dAG mice showed normal suppression of endogenous glucose production but had a significant impairment in glucose clearance when compared to mice receiving vehicle, suggesting that CNS-dAG action impairs peripheral insulin sensitivity. In contrast to the effects observed in wild-type mice, icv-dAG failed to increase FM and induce hyperinsulinemia in GHSR deficient (Ghsr/-)
mice. Taken together, these data demonstrate that dAG is an agonist of GHSR and regulates body adiposity and peripheral glucose metabolism through a central GHSR-dependent mechanism.

Introduction

Acyl ghrelin (AG) regulates multiple aspects of energy metabolism, including feeding and adiposity (Tschop, Smiley et al. 2000; Wren, Small et al. 2000), and is therefore a potential target to treat obesity. Furthermore, ghrelin acts directly on pancreatic islets (Colombo, Gregersen et al. 2003; Wierup, Yang et al. 2004; Qader, Hakanson et al. 2008) to impair glucose-stimulated insulin secretion (Reimer, Pacini et al. 2003; Dezaki, Hosoda et al. 2004; Cui, Ohnuma et al. 2008; Tong, Prigeon et al. 2010) making it a popular target for type 2 diabetes (T2DM) therapies. There is currently only one known ghrelin receptor, namely the growth hormone secretagogue receptor 1a (GHSR). This receptor is expressed in multiple areas of the central nervous system (CNS) where it mediates ghrelin-induced feeding and adiposity. The presence of a fatty acid side-chain (mainly n-octanoic acid) attached to the ghrelin peptide is required for full agonism of GHSR (Kojima, Hosoda et al. 1999). In vitro studies demonstrate that compared to AG, binding of des-acyl ghrelin (dAG) to GHSR, as well as intracellular signaling, are greatly reduced (Kojima, Hosoda et al. 1999; Bednarek, Feighner et al. 2000). Therefore, dAG was initially considered an inactive by-product of ghrelin secretion and degradation. Despite this, multiple reports have suggested that dAG has biological activity including the control of feeding (Chen, Inui et al. 2005; Toshinai, Yamaguchi et al. 2006), body temperature (Inoue, Nakahara et al. 2012), muscle atrophy (Porporato, Filigheddu et al. 2012), and...
2013), glucose-stimulated insulin secretion (Gauna, Kiewiet et al. 2007; Benso, St-Pierre et al. 2012), and lipid metabolism (Thompson, Gill et al. 2004; Giovambattista, Gaillard et al. 2008) attributed to GHSR-independent mechanisms. However, the role of GHSR-mediated dAG action has yet to be directly tested in vivo.

A number of peripherally secreted hormones that act in the CNS to regulate energy metabolism, such as leptin or glucagon-like peptide 1 (GLP-1), also act in the CNS to regulate glucose homeostasis (Obici, Zhang et al. 2002; Sandoval, Bagnol et al. 2008; Morton and Schwartz 2011). Both AG and dAG act directly on pancreatic islet cells to regulate insulin secretion (Gauna, Delhanty et al. 2006), but it is not well established whether they regulate glucose metabolism through central mechanisms. Rats given chronic central administration of AG have elevated fasting insulin levels (Nesic, Stevanovic et al. 2008; Stevanovic, Nesic et al. 2008) which is independent of the hyperphagia induced by ghrelin (Kim, Namkoong et al. 2004) and suggests that AG acts in the CNS to regulate circulating insulin levels. Selective re-expression of GHSR in the hindbrain of mice acts to restore fasting blood glucose levels to control levels, which highlights the importance of central ghrelin receptors to maintain glucose homeostasis (Scott, Perello et al. 2012). Together, these data suggest that AG and GHSR in the CNS regulate glucose metabolism, but whether dAG acts centrally to regulate glucose metabolism has yet to be established.

Using a combination of both in vitro and in vivo techniques, we determined whether GHSR mediates some of the biological actions of dAG. We found that, in contrast to
AG, chronic central administration of dAG does not induce hyperphagia in mice. Interestingly, central infusion of dAG increased adiposity and glucose-stimulated plasma insulin levels to the same extent as AG. These effects cannot be elicited in GHSR-deficient (Ghsr-/-) mice, which demonstrates that dAG is a functional agonist of GHSR in vivo. Collectively, our data suggest that, in addition to multiple GHSR-independent actions, dAG regulates energy storage and glucose metabolism through GHSR in the CNS.

**Materials and Methods**

*Peptide synthesis*

Rat AG and dAG was synthesized using *in situ* neutralization for Boc chemistry, purified by preparative chromatography, and characterized by HPLC and mass spectral analysis, as described previously (Tschop, Smiley et al. 2000).

*Cell culture and transfection*

HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium. DMEM was supplemented with 10 % FBS (PAA Laboratories GmbH), 100 U/ml penicillin, 100 µg/ml streptomycin (Biochrom AG) and 2 mM L-glutamine (Invitrogen). For seeding of HEK-293 cells, 48-well plates were coated with Poly-L-Lysine (Biochrome). 83.3 ng plasmid-DNA/well were transfected using 0.9 µl Metafectene™/well (Biontex).
Measurement of intracellular \( \text{IP}_3 \) formation

Intracellular inositol triphosphate (\( \text{IP}_3 \)) levels were determined using a luciferase reporter assay (Promega). HEK-293 cells were seeded into 48-well plates (5x10^4 cells/well). Equal amounts of receptor-DNA (pcDps) and a reporter construct containing a response element and the firefly luciferase gene under control of the nuclear factor of activated T-cells (NFAT) were co-transfected. Cells were stimulated 2 days after transfection for 6 hours at 37 °C and 5 % CO\(_2\) with AG (1 µM) or with dAG (1 µM) and lysed with 100 µl 1x Passive Lysis Buffer (Promega). IP\(_3\) formation was determined by luciferase activity according to the manufacturer's instructions.

Animals

Male C57BL/6 mice (12 weeks old; Jackson Labs, Bar Harbor, ME) were maintained on a standard chow diet (Teklad, Harlan). After receiving surgery, animals were singly housed and maintained on a 12:12-h light-dark cycle at 22 °C with free access to food and water unless noted otherwise. \( \text{Ghsr}^-/- \) mice and WT control mice were received from Regeneron Pharmaceuticals and bred in our facilities as described previously (Pfluger, Kirchner et al. 2008). All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

Subcutaneous and intracerebroventricular (icv) infusions in mice

For all surgical procedures, mice were anesthetized using 5 % isoflurane in oxygen in an induction chamber and then maintained on 2.5 % isoflurane delivered by a nose
Subcutaneous infusions of AG and dAG were delivered by subcutaneously implanted osmotic-mini pumps (1007D Alzet, Cupertino, CA). For icv infusions, mice were stereotaxically implanted (David Kopf Instruments, Tujunga, CA) with a cannula (brain infusion kit #3, Alzet, Cupertino, CA) placed in the lateral cerebral ventricle of the brain as previously described (Heppner, Chaudhary et al. 2012). A polyethylene catheter attached the cannula to an osmotic mini-pump (1007D Alzet, Cupertino, CA) that was subcutaneously implanted.

**Glucose-stimulated insulin secretion (GSIS)**

Intraperitoneal (ip) GSIS tests were performed by injection of glucose (2 g/kg, 20 % w/v d-glucose, Sigma, in 0.9 % w/v saline) after a 16 h fast. Tail vein blood samples for blood glucose (BG) measurements were collected at time 0, 10, 30, 60 and 120 min after the injection, and were measured using a handheld glucometer (Freestyle Lite). Blood samples for insulin measurements were collected at 0, 10 and 60 min. Plasma insulin was determined using either a radioimmunoassay (Sensitive Rat Insulin RIA; Millipore) or ELISA (Ultra Sensitive Mouse Insulin ELISA Kit; Crystal Chem) as indicated.

**Body composition measurements**

Whole-body composition (fat and lean mass) was measured using NMR technology (Tinsley, Taicher et al. 2004)(EchoMRI-100; Echomedical Systems, Houston, TX). Initial body composition for all experiments was taken the day prior to surgery (d-1). For dose response icv infusion studies (Figure 3.2) body composition was analyzed on d7.
following surgery. For studies involving analysis of GSIS (Figure 3.3, 3.4 and 3.5), body composition was analyzed on d-6 immediately after the GSIS test (~20 h fasted).

*Catheterization for hyperinsulinemic euglycemic clamps*

Mice were catheterized for clamp studies as previously described (Kim, Sandoval et al. 2008). Immediately upon completion of catheterization, animals were implanted with an icv cannula attached to a subcutaneously placed osmotic mini-pump as described above. Following surgery, animals were given subcutaneous injections of buprenorphin (0.28 mg/kg Buprenex; Reckitt Benckiser Healthcare, Richmond, VA), meloxicam (0.25 mg/100 g body weight Metacam; St. Joseph, MO), and 1 mL warm saline.

*Hyperinsulinemic euglycemic clamps*

On day 5 after surgery, a hyperinsulinemic euglycemic clamp was performed as previously described (Kim, Sandoval et al. 2008). Mice were conscious during the entire experimental procedure. Following a 5 h fast, catheter lines were exteriorized and connected to infusion pumps. A 5-µCi bolus of [3-³H]glucose (Perkin Elmer Life Sciences, Boston, MA) was given, followed by a 0.05 µCi/min infusion for 120 min before insulin infusion. At 100 min a blood sample (50 µl) was taken to determine basal glucose and insulin levels as well as basal glucose turnover. The insulin clamp started at 120 min with a continuous infusion of insulin at a rate of 4 mU/kg/min. During the clamp, the [3-³H]glucose infusion rate was increase to (0.1 µCi/min) to maintain constant specific activity. Dextrose (50 g/100 mL) was infused as necessary to maintain euglycemia (~130 mg/dl) on the basis of feedback from frequent arterial glucose
measurements by handheld glucometers (Accu-check Aviva glucometer). Saline-washed erythrocytes previously collected from donor mice were infused throughout the experimental period to prevent a fall of hematocrit. A 12-µCi bolus of 2\[^{14}\text{C}\]deoxyglucose (2\[^{14}\text{C}\]DG) was given at 198 min. Blood samples (20 μl) were taken every 10 min from \(t = 200–240\) min and processed to determine plasma [3-\(^3\text{H}\)]glucose and 2\[^{14}\text{C}\]DG. At the end of the clamp period, mice were euthanized with an injection of sodium pentobarbital. Tissues were collected and stored at -80 °C for further analysis.

**Tracer calculations**

Rates of glucose appearance (Ra), endogenous glucose production (EGP), and glucose utilization were calculated using steady state equations as previously described (Ayala, Bracy et al. 2007). Briefly, endogenous glucose production was calculated by determining the total Ra (this comprises both glucose production and any exogenous glucose infused to maintain the desired glycemic levels) and subtracting the amount of exogenous glucose infused. Tissue specific glucose uptake was calculated from tissue 2-DG content as previously described (Ayala, Bracy et al. 2007).

**Statistical analysis**

Statistical analysis for animal studies was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA). Significance was determined by one-way or two-way ANOVA with either Tukey’s or Bonferroni’s Multiple Comparison post-test and when appropriate by unpaired student’s t-test. For non-linear regression analysis, data sets were fitted using the least square method following the equation
Results

AG and dAG activate GHSR in HEK-293 cells

To determine whether dAG activates GHSR in vitro, we incubated GHSR transfected HEK-293 cells with dAG. We found that 1 µM dAG challenge resulted in a small but significant increase IP₃ turnover above the basal constitutive activity of the receptor (Figure 3.1), which is similar to what other groups have found at this concentration of dAG in stable transfected Chinese hamster ovary cells (Gauna, van de Zande et al. 2007). The IP₃ accumulation induced by AG was approximately 20-fold greater than that induced by dAG (Figure 3.1).

Food intake and body composition following chronic icv infusion of dAG and AG

It is well established that AG increases feeding and adiposity by acting on GHSR expressed in neurons in the brain involved in the control of energy balance. Given that in vitro data suggest that dAG is a weaker agonist of GHSR compared to AG, we hypothesized that infusion of dAG directly into the brain will have similar, but less potent effects as AG on energy metabolism. To test this, we performed chronic central infusion of either AG or dAG at increasing equimolar doses in mice (0, 0.04, 0.2, 1.0 and 5.0 nmol/mouse/day). As expected, 7 days of chronic icv AG infusion increased food intake in a dose dependent manner (EC₅₀ = 0.096 nmol/day, Figure 3.2A). However,
animals receiving chronic icv dAG did not experience an increase in food intake at any
dose used (Figure 3.2A). Interestingly, both AG and dAG increased body weight to a
similar degree (Figure 3.2B). The increase in body weight was due, at last in part, to an
increase in fat mass in comparison with the vehicle-infused control group. Both AG and
dAG caused a similar increase in fat mass in the mice infused with the highest dose
(5.0 nmol/day), although AG was 12.2 fold more potent in comparison with dAG (AG
EC$_{50}$=0.073 vs. dAG EC$_{50}$=0.902 nmol/day, p<0.05) (Figure 3.2C). Changes in lean
mass were similar among all groups (data not shown).

**Effects on glucose metabolism following chronic icv infusion of AG and dAG**

To investigate the central effects on glucose metabolism, mice received chronic icv
infusion of AG or dAG at the highest dose used for our icv dose response experiment
(5.0 nmol/mouse/day). At this dose, AG and dAG had similar effects on body weight and
body composition, but dAG did not alter feeding. Therefore, this dose would give us a
unique opportunity to explore central effects of ghrelin on glucose metabolism that are
dependent as well as independent of changes in food intake. Consistent with our
previous experiment, AG significantly increased food intake, whereas dAG did not alter
food intake (Figure 3.3A). Both ghrelin isoforms increased body weight (Figure 3.3B)
and adiposity (Figure 3.3C) relative to the vehicle-infused control group. Changes in
lean mass were similar among all groups (data not shown). AG and dAG treated mice
showed similar blood glucose excursions during an ip glucose tolerance test (GTT)
(Figure 3D). During the GTT, both treatments significantly increased insulin levels in
comparison with vehicle treated controls, suggesting a higher GSIS (Figure 3.3E).
Effects on energy and glucose metabolism following chronic subcutaneous infusion of AG and dAG

To clarify whether the increases in plasma insulin are due to centrally mediated action or leakage into peripheral tissues, we chronically infused an equimolar amount of AG and dAG (120 nmol/mouse/day) to mice. Consistent with previous reports (Tschop, Smiley et al. 2000), chronic peripheral treatment of AG increased body weight (Figure 3.4B) and fat mass (Figure 3.4C) relative to vehicle-treated controls and had no significant effect on feeding (Figure 3.4A). In contrast to the direct infusion into the brain, chronic subcutaneous administration of a larger dose (24 times the amount given centrally) of dAG did not affect feeding, body weight or fat mass (Figure 3.4A-C) suggesting that the site of action of dAG to increase body weight and fat mass (Figure 3.3) is in the CNS. Lean mass was significantly increased in animals receiving chronic subcutaneously delivered AG relative to vehicle-treated animals (Saline - 7.182 ± 0.5196 g, dAG -5.816 ± 0.3766 g, AG -5.205 ± 0.2920 g; **p< 0.01 Saline vs. AG, one-way ANOVA). To determine whether chronic peripheral treatment of ghrelin alters glucose metabolism, we performed an ip glucose challenge. Blood glucose excursions were similar in all groups (Figure 3.4D). Plasma insulin levels during the ip glucose challenge were elevated in the AG treated group 60 min following injection (Figure 3.4E). Unlike central administration, subcutaneous infusion of dAG, had no effect on glucose-stimulated plasma insulin levels. These data support a centrally mediated action of dAG on glucose-stimulated plasma insulin levels.
Energy and glucose metabolism in Ghsr-/ mice administered with chronic icv dAG

In order to determine the contribution of GHSR action to the effect of central administration of dAG on fat mass and glucose metabolism we performed chronic central infusion of dAG in WT and age-matched Ghsr-/ mice. Consistent with our findings, food intake was not altered due to icv dAG treatment in WT or Ghsr-/ animals (Figure 3.5A). dAG increased body weight and fat mass in WT mice relative to vehicle-treated mice, but had no effect in Ghsr-/ mice (Figure 3.5B,C) demonstrating that this is a GHSR dependent action of dAG. Changes in lean mass were similar among all groups (WT Saline -4.286 ± 0.4610 g, WT dAG -4.092 ± 0.3082 g, Ghsr-/ Saline -4.128 ± 0.3475 g, Ghsr-/ dAG -4.663 ± 0.4586 g; p>0.05, two-way ANOVA). To explore whether the hyperinsulinemia caused by icv dAG is GHSR mediated, we exposed this group of WT and Ghsr-/ mice to an ip glucose-stimulated insulin secretion test. Similar to the effects in Figure 3.3D, administration of an ip glucose bolus did not elicit differences in blood glucose levels in WT or Ghsr-/ mice treated with icv dAG (Figure 3.5D). Consistent with our previous findings, dAG increased plasma insulin levels in WT, but not Ghsr-/ mice during the glucose-stimulated insulin secretion test (Figure 3.5E) demonstrating that this effect of dAG is also GHSR dependent.

Hyperinsulinemic euglycemic clamp in mice treated with chronic icv dAG

The glucose-stimulated hyperinsulinemia induced by icv acyl or dAG treatment could be a result of the development of insulin resistance in these mice. To detect changes in hepatic and/or peripheral insulin sensitivity, we performed a hyperinsulinemic euglycemic clamp in mice that received chronic icv treatment of dAG.
(5.0 nmol/mouse/day). We chose to use dAG in order to limit the confounding factor of hyperphagia that we see with AG and also because we confirmed that the effects of dAG are mediated through acyl ghrelin’s target, GHSR. Blood glucose levels were clamped at similar levels between saline and dAG groups (Figure 3.6A). The exogenous glucose infusion rate necessary to maintain these steady glucose levels was also similar between groups indicating similar rates of whole-body insulin sensitivity (Figure 3.6B). Both saline and dAG treated animals had a similar basal EGP and insulin similarly suppressed EGP during the clamp period indicating that central dAG treatment did not alter hepatic insulin sensitivity (Figure 3.6C). However, while glucose clearance was significantly increased during the clamp in saline treated animals (Figure 3.6D), mice treated with icv dAG had a minor, but significant impairment in their ability to increase peripheral glucose clearance during the clamp (Figure 3.6D). Tissue-specific glucose uptake into soleus, inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), and brown adipose tissue (BAT) was similar between groups (Table 3.1).

Discussion

Cell-based assays show that ghrelin requires esterification with an octanoyl side-chain on the residue serine in position 3 to in order to activate GHSR at concentrations within the nanomolar range (Kojima, Hosoda et al. 1999). Since dAG does not regulate GHSR activity at such concentrations, it has been largely assumed that dAG is not a ligand for GHSR. However, several reports demonstrate that dAG interacts with GHSR at concentrations within the micromolar range (Bednarek, Feighner et al. 2000; Gauna,
van de Zande et al. 2007), suggesting that dAG is a weak GHSR agonist. Our observations in vitro using 1µM of murine dAG on human embryonic kidney cells (HEK-293) transfected with human GHSR are consistent with those reports. The in vitro experiments were performed after overexpression in a heterologous cell system. We use a reporter gene assay which depends on calcium release after IP formation and is therefore comparable to the method used by others (Gauna, van de Zande et al. 2007). Additionally, we evaluated the reporter gene assay against a method where direct IP formation was determined (Winkler, Kleinau et al. 2010). In our test, dAG had a lower stimulation of GHSR activity when compared to an equimolar dose of AG. This is consistent with other studies (Bednarek, Feighner et al. 2000) suggesting that dAG may be a partial GHSR agonist. In contrast, Gauna and colleagues (Gauna, van de Zande et al. 2007) concluded that dAG is actually a full agonist of GHSR since it induced similar maximal receptor stimulation as AG.

Based on the evidence generated in vitro (i.e., dAG is approximately 1000-10000 times less potent than AG), we hypothesized that dAG given at high equimolar doses will mimic some of the biological effects of AG in vivo, including the well-characterized increase in feeding and adiposity. Consistent with that prediction, chronic infusion of dAG directly into the brain failed to elicit the hyperphagia achieved with AG, irrespective of the dose. Interestingly, central infusion of dAG mimicked the effect of AG on adiposity at higher potency (only 12.2 times lower than AG) than could be predicted based on functional assays in vitro. Given that dAG-induced adiposity is completely abolished in mice lacking Ghsr expression, our data suggest that dAG retains significant AG mimetic
activity through GHSR \textit{in vivo}. Furthermore, dAG does not affect adiposity when administered peripherally to WT mice, supporting a centrally mediated action.

In addition to highlighting a central role for dAG in regulating energy metabolism, we recapitulate central AG action on plasma insulin levels (Kim, Namkoong et al. 2004) and further expand on the role of both AG and dAG in the central regulation glucose metabolism. We find that icv infusion of either ghrelin isoform causes hyperinsulinemia in response to a glucose bolus. The glucose-stimulated hyperinsulinemia induced by AG is blunted when AG is infused subcutaneously, whereas the glucose-stimulated hyperinsulinemia induced by dAG is completely ablated when dAG is delivered subcutaneously. The lack of a peripheral action of dAG is consistent with other groups reporting no effect of chronic peripheral dAG treatment on body composition, glucose tolerance or GSIS in chow fed mice (Delhanty, Huisman et al. 2013). Blood glucose excursions are not altered with the increased level of insulin that we find suggesting that insulin sensitivity may be impaired with central ghrelin treatment. Therefore, to measure peripheral insulin sensitivity as well as hepatic glucose production we implemented a hyperinsulinemic euglycemic clamp in mice receiving chronic icv treatment with dAG. We specifically used dAG to avoid the confounding effects of hyperphagia that is induced by icv AG. Furthermore, icv infusion of dAG to \textit{Ghsr} -/- mice failed to induce hyperinsulinemia, indicating a similar receptor mediated action as compared to AG. Basal EGP as well as suppression of EGP during the clamp were similar in icv saline and icv dAG treatment groups indicating that hepatic insulin sensitivity is not impaired. Interestingly, we find that peripheral glucose clearance during the clamp is impaired in
icv dAG treated mice. However, the impairment in glucose clearance is minor, and likely does not account entirely for the increase in plasma insulin levels during a glucose tolerance test. Therefore, our data suggest that AG and dAG may activate central circuits to regulate plasma insulin levels. It is well established that AG acts on GHSR expressed in NPY/AgRP neurons (Nakazato, Murakami et al. 2001). Activation of these neurons results in reduction of the melanocortin system which is associated with hyperinsulinemia (Huszar, Lynch et al. 1997). Whether this mechanism contributes to the increased plasma insulin levels induced by both AG and dAG warrants further investigation.

The increased adiposity and insulin levels with icv dAG treatment in contrast to the lack of effect when dAG is administered peripherally highlights CNS mediated control of peripheral metabolism. Although dAG has been demonstrated to cross the blood-brain-barrier (Banks, Tschop et al. 2002), the amount reaching its central target may have not been potent enough for receptor activation. Interestingly, it has been demonstrated that direct icv injection of dAG does not cross in the brain to blood direction (Banks, Tschop et al. 2002). Therefore, after chronic icv administration, concentrations of dAG may have accumulated and reached a level high enough to activate GHSR. dAG levels increase during calorie restriction (Liu, Prudom et al. 2008; Kirchner, Gutierrez et al. 2009; Zhao, Liang et al. 2010; Yi, Heppner et al. 2012) and interestingly, sequestration of peripherally secreted hormones in the CNS also occurs during calorie restriction (Cashion, Banks et al. 1996). Determining whether metabolic status alters sequestration of dAG in the CNS to levels potent enough to activate GHSR may highlight a role of
peripherally produced dAG. Alternatively, the central effects of dAG despite lack of a peripheral effect may indicate a role for centrally produced ghrelin (Cowley, Smith et al. 2003; Menyhert, Wittmann et al. 2006; Kageyama, Kitamura et al. 2008). Indeed, both AG and dAG are produced in the areas of the brain involved in the control of energy balance (Sato, Fukue et al. 2005). Whether neurally-derived dAG plays a role in the control of energy balance via regulation of GHSR activity remains to be demonstrated. Similarly, it cannot be excluded that factors intrinsic to cell- to-cell interactions in vivo may contribute to a relative increase in GHSR-mediated dAG biological activity as reported here.

A growing area of interest is the ability of GHSR to heterodimerize with other G-protein coupled receptors (GPCRs) (Rediger, Piechowski et al. 2011; Kern, Albarran-Zeckler et al. 2012; Park, Jiang et al. 2012; Schellekens, van Oeffelen et al. 2012). Formation of these heteromers modifies GHSR basal activity as well as AG action (Rediger, Piechowski et al. 2011). Furthermore, the amount of ghrelin agonist present influences GHSR heterodimer formation as well as coupling to downstream intracellular signaling systems (Park, Jiang et al. 2012). Investigating whether dAG modulates GHSR heterodimerization with other GPCRs could help elucidate the function and molecular mechanisms of endogenous dAG.

Collectively, our data demonstrate that dAG increases adiposity and plasma insulin levels through the interaction with GHSR in the CNS and suggest that dAG may be functional endogenous agonist of GHSR. Our data highlight the complexity of the
ghrelin system and implicate that further studies are necessary to fully understand each component of this system.

These data highlight novel neuropharmacological roles of dAG and AG in regulating glucose and energy metabolism. Our final aim was to determine the endogenous role of the GOAT-Ghrelin system in regulating glucose tolerance by using genetically modified mouse models in combination with acute pharmacological interventions.
Figure 3.1: Effect of AG and dAG on IP₃ formation in HEK-293 cells expressing human GHSR. Functional *in vitro* assays were performed in transiently transfected HEK-293 cells to investigate dAG (1 µM) and AG (1 µM) Gq/11 mediated IP₃ formation was determined via a reporter construct containing a response element and the firefly luciferase gene under control of the nuclear factor of activated T-cells (NFAT). All data indicate a fold increase of the GFP transfected cells (MOCK) basal activity 6481 ± 1937 rlu (relative light units for IP₃, which was set as 1). Data are means ± SEM out of three experiments performed in sixtuples for Gq/11 activation. Analysis of variance was done by an unpaired two-tailed t test.
Figure 3.2: Effect of chronic icv dAG or AG infusion on energy metabolism.

C57/BL6 mice were given icv infusion of saline, dAG or AG for 7 days. AG and dAG were infused using increasing doses (0.04, 0.2, 1.0 and 5.0 nmol/mouse/day). AG dose-dependently increased food intake whereas dAG had no effect on food intake (A). Both AG and dAG increased body weight (B) and fat mass (C) in a dose-dependent manner. n= 6-10 animals per group.
Figure 3.3: Effect of chronic icv dAG or AG infusion on energy and glucose metabolism. C57/BL6 mice were given icv infusion of saline, dAG or AG for 6 days (5 nmol/mouse/day). AG significantly increased food intake (A; *p<0.05 vs. saline, one-way ANOVA). Icv infusion with AG or dAG increased body weight (B; *p<0.05 vs. saline, one-way ANOVA) and fat mass relative to saline-treated control animals (C; *p<0.01, ***p<0.001 vs. saline, one-way ANOVA). Neither AG nor dAG icv infusion altered ip glucose tolerance (D). Plasma insulin levels were measured by RIA and were elevated in mice treated with AG or dAG (E; *p<0.05 dAG vs. saline, ##p<0.01 AG vs. saline; two-way ANOVA). n= 8-9 animals per group.
Figure 3.4: Effect of chronic subcutaneous dAG or AG infusion on energy and glucose metabolism. C57/BL6 mice were subcutaneously implanted with mini-pumps that infused AG or dAG for 6 days (120 nmol/mouse/day). Neither ghrelin compound had a significant effect on food intake (A). AG increased body weight (B) and fat mass relative to saline-treated controls (C), whereas dAG had no effect. Neither AG nor dAG infusion altered ip glucose tolerance (D). Plasma insulin levels were measured by RIA and were elevated in icv-AG treated mice at 60 minutes following glucose injection (E). (*p<0.05, ***p<0.001 AG vs. saline, one-way ANOVA) (##p<0.01 AG vs. saline; two-way ANOVA). n= 8-9 animals per group.
Figure 3.5: Effect of chronic icv dAG infusion on energy and glucose metabolism in Ghsr -/- mice. Ghsr-/− and age-matched WT mice were given icv infusion of saline or dAG ghrelin for 6 days (5 nmol/mouse/day). Icv dAG infusion did not alter feeding in WT or Ghsr-/− animals (A). Body weight (B) and fat mass (C) were increased in WT mice centrally treated with dAG relative to WT saline-treated controls. Icv dAG infusion had no effect on body weight (B) or fat mass (C) in Ghsr-/− mice. Icv dAG did not altered ip glucose tolerance in either WT or Ghsr-/− animals (D). Plasma insulin levels were measured by ELISA and were elevated in WT mice treated with icv dAG (E) compared to icv saline WT mice. Icv dAG treatment did not alter glucose-stimulated plasma insulin levels in Ghsr-/− mice compared to icv saline treated Ghsr-/− mice (E). (**p<0.01, ***p<0.001, WT saline vs. WT dAG; two-way ANOVA). n= 7-8 animals per group.
Figure 3.6: Effect of chronic icv dAG infusion on peripheral glucose homeostasis during a hyperinsulinemic euglycemic clamp. Mice received chronic icv infusion of dAG for 5 days prior to a hyperinsulinemic euglycemic clamp. Blood glucose levels were clamped at similar levels in saline and dAG treated mice (A). Exogenous glucose infusion rate was similar in saline and dAG treated mice (B). Basal EGP and suppression of EGP during the hyperinsulinemic euglycemic clamp were similar in saline and dAG treated mice (C). Animals treated with dAG had impaired insulin-mediated glucose disposal (D). *p<0.05 saline vs. dAG; t-test. n= 9-10 animals per group.
Table 3.1: Effect of chronic icv dAG infusion on tissue specific glucose uptake during a hyperinsulinemic euglycemic clamp. No difference in glucose uptake in BAT, iWAT, eWAT or soleus was detected in icv dAG treated mice compared to saline treated controls. N =4-7 animals per group
CHAPTER 4

The endogenous role of GOAT in regulating glucose tolerance: effect of nutritional status and age
Abstract

Ghrelin is an acylated gastrointestinal peptide that promotes a positive energy balance. The enzyme ghrelin O-acyltransferase (GOAT) esterifies an $n$-octanoic acid to the peptide, thereby enabling ghrelin to bind and fully activate the ghrelin receptor. Ghrelin has been implicated to regulate glucose metabolism. However, the exact role that endogenous GOAT plays in regulating glucose tolerance is unclear. Mice that lack $\text{Goat}$ ($\text{Goat}^{-/-}$) produce des-acyl ghrelin (dAG), but are unable to produce acyl ghrelin (AG). We find that glucose tolerance is improved in $\text{Goat}^{-/-}$ mice under 16h fasting conditions but not under 2h fasting conditions. Furthermore, young $\text{Goat}^{-/-}$ mice have improved 16h fasting glucose tolerance, whereas aged $\text{Goat}^{-/-}$ mice have a similar 16h and 24h fasting glucose tolerance as age-matched WT controls. To clarify whether the improved glucose tolerance in $\text{Goat}^{-/-}$ mice is a result of the absence of AG or a result of unopposed dAG action we exogenously administered AG or dAG to $\text{Ghrelin}^{-/-}$ mice prior to the glucose tolerance test (GTT). Administration of AG (60nmol ip) 30 minutes prior to the GTT impaired glucose tolerance, whereas administration of dAG (60nmol ip) had no effect on glucose tolerance. These data indicate that the lack of AG in $\text{Goat}^{-/-}$ mice improves glucose tolerance, and moreover, calorie restriction and age are critical determinants for revealing the role of GOAT in regulating glucose tolerance.
Introduction

Ghrelin is a gastrointestinal polypeptide that regulates both energy and glucose metabolism (reviewed in (Heppner, Tong et al. 2011; Kirchner, Heppner et al. 2012)). Acylation of ghrelin with an n-octanoic acid on its serine 3 residue is required for full agonism of the ghrelin receptor (GHSR) (Kojima, Hosoda et al. 1999). The enzyme that provides the acyl modification is ghrelin O-acyltransferase (GOAT). Acute exogenous administration of acyl ghrelin (AG) reliably impairs glucose tolerance through the inhibition of insulin secretion (Dezaki, Hosoda et al. 2004; Tong, Prigeon et al. 2010). Conversely, acute pharmacological inhibition of the enzyme GOAT improves glucose tolerance by enhancing insulin secretion in mice (Barnett, Hwang et al. 2010). However, inconsistent findings in genetically modified animals have made it difficult to define the role of the endogenous GOAT in regulating glucose metabolism.

Goat-/- mice have improved glucose tolerance when fed a standard chow diet (Sun, Asnicar et al. 2006; Zhao, Liang et al. 2010). In contrast, other groups have reported no differences in glucose tolerance in chow-fed Goat-/- mice (Kirchner, Gutierrez et al. 2009). A major difference in these studies was the fasting period prior to the glucose tolerance test (GTT) (6h vs. 18h). Circulating ghrelin levels as well as Goat gene expression are regulated by fasting (Liu, Prudom et al. 2008; Kirchner, Gutierrez et al. 2009). Therefore, the length of time that animals are calorie restricted prior to the GTT may be critical for determining differences in glucose tolerance in Goat-/- mice. Some reports indicate that ghrelin acylation by the enzyme GOAT is essential for maintaining blood glucose levels during calorie restriction (Zhao, Liang et al. 2010; Li, Sherbet et al.
Goat-/- mice become severely hypoglycemic to the point of death when placed on a 60% calorie restricted diet (Zhao, Liang et al. 2010). The authors attribute this to the lack of AG induced growth hormone (GH) secretion (Zhao, Liang et al. 2010). The authors demonstrate that when calorie restricted Goat-/- mice are treated with either AG or GH, glycemic levels are restored to the level of WT mice (Zhao, Liang et al. 2010). However, the role of ghrelin induced GH secretion in maintaining blood glucose levels during severe calorie restriction is not supported by other groups. Although GH levels significantly rise during calorie restriction, adult-onset isolated GH deficient (AOiGHD) mice maintain similar blood glucose levels as their controls during calorie restriction (52-weeks-old) (Gahete, Cordoba-Chacon et al. 2012) indicating that GH is not essential for maintaining glycemia during calorie restriction. Furthermore, our group used the same calorie restriction paradigm as Zhao et al., and we found that mice lacking ghrelin, Ghsr, or Goat were able to maintain a similar blood glucose level as their WT counterparts even when fat mass levels reached ~0% (Yi, Heppner et al. 2012). One hypothesis for the discrepancy among these reports could be due to the significant difference in age of the mice used. Zhao et al. used young 8-week-old male mice that are still in the growing phase, whereas Yi et al. and Gahete et al. used mature (7-13 months) adult mice. In rodents, gastric ghrelin gene expression has been shown to increases rapidly after birth and then slowly decline with age (Liu, Yakar et al. 2002). Furthermore, circulating ghrelin reaches peak levels at 8-weeks of age in male mice (Liu, Yakar et al. 2002). Therefore, the changes in circulating ghrelin levels during aging may be causing the discrepancy in reports in the literature characterizing ghrelin’s role in regulating glucose metabolism.
Taken together, the use of different fasting paradigms as well as different ages of genetically altered mice could be important factors leading to inconsistent reports in the literature that characterize the endogenous role of the ghrelin system in regulating glucose metabolism. We aimed to examine the impact of both calorie restriction and age on glucose tolerance in Goat-/- mice.

**Material and Methods**

**Animals**

Male Ghrelin-/- mice, Goat-/- mice and Ghrelin-/-Ghsr double knockout (dKO) mice were generated as previously described (Wortley, Anderson et al. 2004; Gutierrez, Solenberg et al. 2008; Pfluger, Kirchner et al. 2008; Kirchner, Gutierrez et al. 2009). Mice were maintained on a standard chow diet (Teklad, Harlan), and a 12:12-h light-dark cycle at 22 °C with free access to food and water unless noted otherwise. All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Cincinnati.

**Intraperitoneal glucose tolerance test (ipGTT)**

Animals were fasted for 2h or 16h prior to the ipGTT. A bolus of glucose (2 g/kg, 20 % w/v d-glucose, Sigma, in 0.9 % w/v saline) was injected at 09:00 for all experiments. Tail vein blood samples for blood glucose (BG) measurements were collected at time 0, 10, 30, 60 and 120 min after the injection, and were measured using a handheld glucometer (Freestyle Lite).
Intraperitoneal glucose tolerance test (ipGTT) with acyl ghrelin (AG) and des-acyl ghrelin (dAG) pre-treatment

Animals were fasted 16h prior to injection. At 09:00, mice (9-months-old) were injected with saline, AG (60nmol/mouse, ip), or dAG (60nmol/mouse, ip). After 30 minutes, a bolus of glucose (2 g/kg, 20 % w/v d-glucose, Sigma, in 0.9 % w/v saline, ip) was injected. Tail vein blood samples for blood glucose (BG) measurements were collected at time 0, 10, 30, 60 and 120 min after the injection, and were measured using a handheld glucometer (Freestyle Lite).

Statistical analysis

Statistical analysis for animal studies was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA). Significance for glucose tolerance tests was determined by two-way ANOVA with Bonferroni’s Multiple Comparison post-test. Significance for body weight comparison was determined by unpaired student’s t-test. All results are given as means ± SEM. Results were considered statistically significant when p < 0.05.

Results

Glucose tolerance in Goat-/- mice under fed and fasting conditions.

To determine whether calorie restriction impacts glucose tolerance in mice lacking Goat, we subjected Goat-/- and age-matched WT control mice to an ipGTT under fed and fasting conditions. The age of the mice was a similar age that was used in the Zhao et al. study (Zhao, Liang et al. 2010). Body weights were similar in WT and Goat-/-
animals under fed conditions (WT 29.36 ± 0.52g vs. *Goat-/-* 28.14 ± 0.98; p=0.27, t-test). We did not detect differences in glucose tolerance when mice were fasted 2h prior to an ipGTT (Figure 4.1A). The same cohort of mice was subjected to an ipGTT following an overnight (16h) fast. Fasting body weights were similar in WT and *Goat-/-* mice (WT 26.18 ± 0.39g vs. *Goat-/-* 25.41 ± 0.85g; p=0.41, t-test). *Goat-/-* mice exhibit improved glucose tolerance compared to WT controls under 16h fasting conditions (Figure 4.1B). These data indicate that calorie restriction is essential for revealing the glucose tolerant phenotype in *Goat-/-* mice.

**Glucose tolerance in aged *Goat-/-* mice.**

To determine whether age influences the glucose tolerant phenotype in *Goat-/-* mice, the same cohort of *Goat-/-* mice was subjected to an ipGTT when they reached a similar age as in the study by Yi et al (Yi, Heppner et al. 2012). After an overnight fast, mice were exposed to an ipGTT. Fasting body weights were similar between aged WT and *Goat-/-* mice (WT 32.38 ± 0.65g vs. *Goat-/-* 30.81 ± 0.66g; p=0.11, t-test). No difference in glucose tolerance was observed between WT and *Goat-/-* animals (Figure 4.2A). It is possible that a 16h fast was a long enough fast to challenge the systems of these older mice. Therefore, to further challenge this older set of mice, animals were exposed to an ipGTT following a 24h fast (Figure 4.2B). Body weights were similar following a 24h fast (31.57 ± 0.48g vs. *Goat-/-* 31.24 ± 0.38g; p=0.61, t-test). No difference in glucose tolerance was noted between the groups under 24-h fasting conditions (Figure 4.2B).
Acute effect of exogenous acyl and des-acyl ghrelin on glucose tolerance

The improvement in glucose tolerance in Goat-/− mice has been attributed to the lack of AG inhibition of insulin secretion. However, some groups believe that the ratio of acyl/des-acyl ghrelin plays an important role in regulating glucose metabolism (Delhanty and van der Lely 2011). Therefore, it is possible that the effect we see in Goat-/− mice is due to the action of dAG in the absence of AG. To provide evidence that the improvement in glucose tolerance observed in Goat-/− mice is due to the lack of AG and not due to the unopposed action of dAG, we administered an acute dose of either AG or dAG (60nmol/mouse, ip) to Ghrelin-/− mice 30 minutes prior to an ipGTT. We used Ghrelin-/− mice to avoid any interaction with endogenously produced ghrelin. Animals treated with dAG have similar blood glucose excursions as animals treated with saline (Figure 4.4A). Ghrelin-/− mice treated with AG have impaired glucose tolerance compared to saline and dAG treated mice (Figure 4.4A). To test whether this effect of AG is mediated through GHSR, we repeated this experiment in Ghrelin-Ghsr double knockout (dKO) mice (Figure 4.4B). All treatment groups exhibit similar blood glucose excursions indicating that the impairment in glucose tolerance induced by AG in Figure 4.4A is mediated through GHSR.

Discussion

The endogenous role of the GOAT in regulating glucose metabolism has been difficult to characterize due to inconsistent data in the literature. Our data demonstrate that the glucose tolerance phenotype in Goat-/− mice is dependent on nutritional status and age. Consistent with previous reports (Zhao, Liang et al. 2010), we find that Goat-/− mice
have improved glucose tolerance under 16h fasting conditions. When animals are 
fasted only 2h prior to an ipGTT, no difference in glucose tolerance is observed 
between WT and \textit{Goat-/-} mice. This is consistent with reports showing that there is no 
difference in glucose tolerance between \textit{Goat-/-} and WT mice when a shorter fasting 
paradigm (6h) prior to the GTT is implemented (Kirchner, Gutierrez et al. 2009). Our 
data indicate that the different fasting paradigms led to different conclusions about the 
role of GOAT in regulating glucose tolerance. Ghrelin acylation is influenced by fasting 
(Liu, Prudom et al. 2008), and therefore, it is logical that calorie restriction is an 
important factor in determining differences in glucose homeostasis in \textit{Goat-/-} animals.

Ghrelin can promote GHSR coupling through the \text{Ga}_{i/o} signaling pathway in pancreatic 
\(\beta\)-cell islets which leads to the inhibition of insulin secretion (Dezaki, Kakei et al. 2007). 
The reason that GHSR couples to a \text{Ga}_{i/o} instead of a \text{Ga}_{q/11} pathways in the \(\beta\)-cell was 
recently explored by Park et al. (Park, Jiang et al. 2012). This group demonstrated that 
GHSR heterodimerizes with somatostatin receptor 5 (SST5) which promotes GHSR 
coupling to \text{Ga}_{i/o}. The GHSR:SST5 heteromer formation is influenced by the ratio of 
[ghrelin]/[SST] present. Under fasting conditions, the [ghrelin]/[SST] ratio is high and 
GHSR couples with \text{Ga}_{i/o} leading to an inhibition of insulin secretion. However, under 
fed conditions, the ratio of [ghrelin]/[SST] is low and leads to the destabilization of the 
GHSR:SST5 heteromer and GHSR no longer couples to \text{Ga}_{i/o}. These data could 
explain why the glucose tolerant phenotype in \textit{Goat-/-} mice is only apparent under 
fasting conditions, although further investigation is required.
We also find that age is a critical determinant for the glucose tolerant phenotype in both Goat-/- mice. As the animals age, the improvement in glucose tolerance that was observed in the Goat-/- animals is no longer apparent. The significantly older age as well as a shorter fasting paradigm (6h) could be the reason that some reports indicate that Ghrelin-/-, Ghsr-/-, and Ghrelin-/-Ghsr-/- dKO mice have similar glucose tolerance to WT controls (Pfluger, Kirchner et al. 2008). These data suggest that the ghrelin system does not play a significant role in the regulation of glucose homeostasis in mature adult animals. Therefore, the discrepancy between the Zhao et al. study and the Yi et al. study may have been due to the different ages of animals used. However, whether our data investigating glucose tolerant phenotypes in Goat-/- mice will translate to glucose regulation during calorie restriction still needs to be determined. Ghrelin expression in pancreatic islets is highest and outnumbers expression in the stomach during the early developmental period (Chanoine and Wong 2004). Gastric ghrelin expression in the fetus is low but increases rapidly after birth, reaching adult levels by 3-5 weeks of age (Hayashida, Nakahara et al. 2002). In contrast, pancreatic ghrelin expression declines after birth and very low expression is detected in adult human pancreatic islets (Wierup, Svensson et al. 2002; Andralojc, Mercalli et al. 2009). These data suggest that ghrelin in the pancreas may play a more predominant role in regulating glucose metabolism at early stages in development. Furthermore, changes in ghrelin gene expression during aging may indicate that ghrelin plays different tissue specific roles at different stages of life.
We also find that AG impairs glucose tolerance in animals that have intact GHSR signaling, whereas AG has no effect on glucose tolerance in animals that lack GHSR indicating a GHSR mediated action. We find that dAG has no acute effect on glucose tolerance in animals lacking ghrelin. This suggests that the improvement in glucose tolerance in Goat-/- mice is due to the lack of AG signaling through GHSR.

Interestingly, Ghsr-/- mice have similar glucose tolerance as WT control mice at a young age, but are resistant to developing age-related glucose intolerance (Lin, Saha et al. 2012). The difference in glucose tolerant phenotypes between Ghsr-/- and Goat-/- mice is intriguing. Whether the difference in phenotype is due to the constitutive activity of the receptor (Holst, Cygankiewicz et al. 2003) or altered Ghsr gene expression during aging warrants further investigation.

Taken together, our data demonstrate that calorie restriction and age are critical factors that impact the role of GOAT in mediating glucose tolerance. Importantly, the age-dependent effect may account for the discrepancies of the published calorie restriction experiments (Zhao, Liang et al. 2010; Yi, Heppner et al. 2012). Further studies are needed to more clearly define each component of the GOAT/Ghrelin/GHSR system in order to fully understand the system’s role in regulating glucose metabolism.
Figure 4.1: Improved fasting glucose tolerance in young Goat-/- mice. (A)

Glucose tolerance is improved in Goat-/- mice compared to age-matched WT control mice (2-3 months old) following a 16h fast (2g/kg BW of 25% D-glucose given ip). (*p<0.05, **p<0.01 WT vs. Goat-/-, two-way ANOVA) (B) Glucose tolerance is similar in WT and Goat-/- mice fasted for 2h. n=7-8 animals per group.
Figure 4.2: Improved glucose tolerance in Goat-/- mice is age dependent. (A) Glucose tolerance after a 16h fast is similar in WT and Goat-/- mice at 7-8 months of age. (B) Glucose tolerance after a 24h fast is similar in WT and Goat-/- mice at 7-8 months of age. n=7-8 animals per group.
Figure 4.3: Acute effect of exogenous AG and dAG on glucose tolerance in Ghrelin-/− and Ghrelin-/− Ghsr-/− double knockout mice. (A) Administration of AG (60nmol, ip) to Ghrelin-/− mice 30 min prior to ipGTT impairs glucose tolerance whereas dAG (60nmol, ip) has no effect on glucose tolerance (n=5-6 animals per group) (*p<0.05, **p<0.01 saline vs. AG, two-way ANOVA). (B) Administration of AG (60nmol, ip) or dAG (60nmol, ip) to Ghrelin-/−Ghsr-/− dKO mice 30 min prior to ipGTT has no effect on glucose tolerance (n=7-8 animals per group).
CHAPTER 5

General Discussion
Summary of dissertation research

These data provide novel insight into the role that ghrelin acylation has on modulating ghrelin action on energy and glucose metabolism. These data show that the fatty acid side-chain that acylates ghrelin modifies ghrelin activation of GHSR in vitro as well as ghrelin action on energy and glucose metabolism in vivo. We find that GHSR activation by ghrelin isoforms with varying FA-side chain length in vitro does not always predict potency of action in vivo. Furthermore, we find that the most abundant forms of endogenously produced ghrelin, C8-acylated ghrelin (AG) and des-acyl ghrelin (dAG), both act in the CNS to increase glucose-stimulated plasma insulin levels without altering glucose tolerance. Many reports demonstrate that dAG has a wide variety of biological actions (Thompson, Gill et al. 2004; Chen, Inui et al. 2005; Toshinai, Yamaguchi et al. 2006; Gauna, Kiewiet et al. 2007; Giovambattista, Gaillard et al. 2008; Benso, St-Pierre et al. 2012; Inoue, Nakahara et al. 2012; Porporato, Filigheddu et al. 2013). Due to weak agonism of GHSR, the effects of dAG have been mainly attributed to GHSR independent actions (Kojima, Hosoda et al. 1999; Bednarek, Feighner et al. 2000). Using loss of function mouse models, we find that dAG acts through GHSR in the CNS to induce adiposity and increase glucose-stimulated plasma insulin levels. In contrast to chronic central action, acute peripheral administration of AG acts to impair glucose tolerance, whereas dAG has no acute peripheral effect on glucose tolerance which has also been reported by other groups (Broglio, Gottero et al. 2004; Dezaki, Hosoda et al. 2004). The acute peripheral actions of AG are consistent with loss of function models. Specifically, Goat-/- and Ghrelin-/- mice, which both lack circulating AG have improved glucose tolerance (Sun, Asnicar et al. 2006; Zhao, Liang et al. 2010). Our data
demonstrate that the improved glucose tolerance in Goat-/− animals is dependent on calorie restriction and age. Taken together, our data demonstrate that modifying the FA side-chain that acylates ghrelin provides a unique approach to modulate the biological action of ghrelin. Furthermore, our data demonstrate that regulation of glucose tolerance by endogenous GOAT, and therefore ghrelin acylation, is dependent on nutritional status and age. Taken as a whole, these data highlight the complexity of the ghrelin system.

Modification of the acyl side chain of ghrelin as a novel approach to modulated ghrelin activity

The data from the current studies indicate that ghrelin activity can be modulated by altering the length of the FA side-chain. Importantly, our data highlight that the in vitro read-out for GHSR activation does not always predict in vivo action. For example, C12-acylation of ghrelin causes a right shift in the dose response curve for IP accumulation in COS-7 cells transiently transfected with GHSR indicating a decreased potency to activate GHSR (Heppner, Chaudhary et al. 2012). However, chronic icv administration of C12-acylated ghrelin induces the same degree of adiposity as native C8-acylated ghrelin and this effect is dependent on GHSR action. Similarly, we find that dAG is a significantly weaker agonist of GHSR in HEK-293 cells as compared to AG, which is consistent with findings from other groups (Kojima, Hosoda et al. 1999; Bednarek, Feighner et al. 2000; Gauna, van de Zande et al. 2007). We find that dAG has much higher potency of action on adiposity in mice than could be predicted from these cell-based assays. Moreover, we demonstrate that dAG requires GHSR to mediate its
effects on adiposity. In addition, ghrelin acylated with a C16 FA has similar dose response curve for IP accumulation in COS-7 cells transiently transfected with GHSR as compared to native C8-acylated ghrelin. As predicted from the dose response curves, both compounds increase fat mass to a similar level when given chronically icv. However, chronic daily subcutaneous injection of C16-acylated ghrelin has a higher maximal effect on body weight and adiposity as compared to C8-acylated ghrelin. Most of the in vitro assays, including those presented here, measure GHSR activation in cells based on IP3 accumulation or IP3-coupled mobilization of intracellular calcium. Although these are well established assays for predicting in vivo action of C8-acylated ghrelin, our data suggest that these assays do not always predict in vivo action of dAG or ghrelin acylated with side-chains other than C8. In accordance with this, one report investigated the minimal amino acid sequence and structure of ghrelin that was required for GHSR activation as determined by IP3-coupled mobilization of intracellular calcium (Bednarek, Feighner et al. 2000). The first 4-5 amino acids as well as the n-octanoic acid esterified to the serine3 residue were required for GHSR activation. However, another group showed that peripheral administration, even at large doses, of these short peptide fragments did not promote GH secretion in rats. The authors suggested that calcium mobilization may not fully reflect the activation of all the signal transduction systems required to activate the endogenous receptor that lead to the subsequent biological read-out (i.e. GH secretion) (Torsello, Ghe et al. 2002). Increasing evidence suggests that GHSR heterodimerizes with multiple other GPCRs, which can modify potency of action of the GHSR ligand and regulate the basal activity of GHSR (Rediger, Piechowski et al. 2011; Park, Jiang et al. 2012; Schellekens, van Oeffelen et al. 2012).
Depending on the cells used to test GHSR activation, these additional GPCRs may not be present and therefore the in vitro system may not reflect the full activity of the ligand that is observed in a living organism. Furthermore, GHSR couples to other G-proteins such as Ga\(_{i/o}\), and therefore measuring IP accumulation does not account for these other signaling cascades (Dezaki, Kakei et al. 2007; Park, Jiang et al. 2012). Lastly, the basal constitutive activity of GHSR in a living organism may not reflect the same level of activity when the receptor is transfected into cells. Therefore, if GHSR basal activity in transfected cells overestimates the activity of the receptor in vivo, this may mask the activity of partial agonists in the in vitro assay, which may be the case for dAG activation of GHSR. Taken together, the current data emphasize that the in vivo GHSR signaling system is complex, and therefore, conclusions of GHSR mediated activity of GHSR ligands based on in vitro assays should be made with caution. Importantly, these data implicate that screening for effective GHSR agonists requires a combination of in vitro and in vivo testing.

Does length matter?

Our data indicated that increasing the FA-side chain length of ghrelin does not necessarily increase the effectiveness of centrally administered ghrelin on adiposity. For example, at high equimolar doses (5nmol/day), icv dAG increases adiposity in mice to the same extent as C8-acylated ghrelin. However, acylation of ghrelin with a longer FA side-chain such as C14 actually decreases the effectiveness of this ghrelin isoform to induce adiposity when it is administered icv. Therefore, it is not simply increasing the FA side-chain that predicts increasing effectiveness of centrally administered ghrelin.
When does length matter? We find that the length of the FA that acylates ghrelin plays a critical role in regulating the effectiveness of peripherally administered ghrelin.

Chronic subcutaneous administration of dAG, even at extremely high doses (120nmol/day = 400µg/day) does not alter adiposity, food intake or body weight in mice. In contrast, chronic subcutaneous administration of C16-acylated ghrelin increases body weight and adiposity to a higher degree as compared to C8-acylated ghrelin. Although we did not test the peripheral administration of all of the ghrelin isoforms, it is clear that C0<C8<C16 in terms of effectiveness on body weight and adiposity. Therefore, our data suggest that increasing the FA-side chain length of ghrelin increases the maximal effectiveness of peripherally administered ghrelin. From our data it is not clear as to why increasing the FA side-chain of ghrelin increases the effectiveness of peripherally administered ghrelin. Ghrelin acts on GHSR located in the ARC of the CNS to modulate adiposity and body weight. Therefore, a longer FA side-chain length may aid in the delivery of the ghrelin molecule to its centrally located target. One hypothesis that we had was that C16-acylation increases the stability of the ghrelin molecule by increasing the resistance to circulating deacylation enzymes that rapidly cleave the FA side-chain. We provide evidence that this is not the case by showing that systemic administration of a stabilized C8-acylated ghrelin compound (SC8) containing a non-hydrolyzable C8 FA attached to the ghrelin peptide has similar effects on body weight and adiposity as native C8-acylated ghrelin (non-stabilized). Another possibility for the increased effectiveness of peripherally administered ghrelin with longer FA side-chains is that the longer FA increases the hydrophobicity of the ghrelin molecule, which may allow for easier transport across the BBB. However, mechanisms that explain this
hypothesis require further clarification. Another possibility is that ghrelin acylated with longer chain FA may more readily bind to serum proteins. AG binds to circulating carrier proteins, whereas dAG does not (Patterson, Murphy et al. 2005; De Vriese, Hacquebard et al. 2007). Similarly, long-chain FAs bind to serum albumin and acylated peptides such as liraglutide allowing the peptides to remain in circulation for a longer period of time (Hamilton, Era et al. 1991). Furthermore, the acyl side-chain of ghrelin acts to anchor the peptide molecule to the membrane of a cell (Grossauer, Kosol et al. 2010). Taken together, the data from the literature suggest that the increased effectiveness of ghrelin acylated with long hydrophobic FA side-chains may be a result of the FA side-chain of ghrelin binding to circulating carrier proteins or acting as membrane anchor. Together, this may allow the ghrelin peptide molecule to more effectively reach its target receptor. However, further clarification is needed to define these phenomena as the causative factors that enhance the effectiveness of peripherally administered ghrelin containing longer FA side-chains. Collectively, our current data indicate that increasing the FA side-chain length of ghrelin is a novel approach to increase the effectiveness of peripherally administered ghrelin.

Tissue specific production and action of AG, dAG, and ghrelin isoforms

As stated above, these data demonstrate that altering the FA side-chain length of ghrelin can modify its effectiveness on adiposity. This may be a novel way to modulate the effectiveness of ghrelin used for pharmacotherapies. However, the question still remains as to whether these ghrelin isoforms have an endogenous role in regulating energy metabolism. Although C8-acylated ghrelin is the most abundant form of
endogenously produced acyl ghrelin, ghrelin acylated with a C10 and C10:1 have also been found in circulation. Furthermore, supplementing the diet with C6, C7, or C10 FAs results in the production of ghrelin acylated with these FAs (Nishi, Hiejima et al. 2005; Kirchner, Gutierrez et al. 2009). Ghrelin acylated with a C16 FA was not detected in the stomach or in circulation when mice were fed a diet rich in C16 FA (Nishi, Hiejima et al. 2005). However, preproghrelin and GOAT mRNA are co-expressed in a number of other tissues including the pancreas and the brain (Gutierrez, Solenberg et al. 2008; Gahete, Cordoba-Chacon et al. 2010; Lim, Kola et al. 2011). Whether these tissues are able to produce various ghrelin isoforms has yet to be determined.

Central versus peripheral regulation of glucose metabolism

A large body of literature provides clear evidence that the CNS is involved in regulating peripheral glucose metabolism (Schwartz and Porte 2005; Sandoval, Obici et al. 2009). Many peripherally secreted hormones act upon peripheral tissues as well as in the CNS to regulate glucose homeostasis. Insulin, which is secreted from the β-cells, is well known for its action to promote glucose uptake in the periphery by acting directly on peripheral tissues. Insulin receptors are also located in the CNS and centrally administered insulin suppresses EGP (Obici, Zhang et al. 2002). Glucagon-like peptide 1 (GLP-1) is released from the L-cells of the intestine as nutrients are ingested. GLP-1 acts on GLP-1Rs in pancreatic β-cells to promote insulin secretion which helps to regulate blood glucose excursions following meal consumption (Scrocchi, Brown et al. 1996). GLP-1Rs are also located in the CNS and activation of these receptors regulates glucose homeostasis. Icv injection of GLP-1 increases glucose-stimulated
insulin secretion, and injection of GLP-1 into the ARC reduces EGP during a hyperinsulinemic euglycemic clamp (Sandoval, Bagnol et al. 2008). Reports in the literature indicate that both AG and dAG ghrelin act directly on pancreatic islet cells to regulate insulin secretion (Colombo, Gregersen et al. 2003; Wierup, Yang et al. 2004; Gauna, Delhanty et al. 2006). Our data provide novel evidence that, like many other peripherally secreted hormones that regulate energy balance, AG and dAG ghrelin act in the CNS to regulate glucose metabolism. Our data demonstrate that although dAG is a weak agonist of GHSR, it acts through GHSR in the CNS to regulate plasma insulin levels as well as peripheral insulin sensitivity. A point of interest that is in need of further clarification in the future is the observation that chronic icv administration of AG increases glucose-stimulated plasma insulin levels whereas acute peripheral administration of AG impairs glucose tolerance by inhibiting insulin secretion. The reason for these differences may be due to acute versus chronic action of AG or a central versus peripheral effect of AG. We find that chronic subcutaneous administration of AG does not have as strong of an effect as icv treatment, but tends to increase glucose-stimulated plasma insulin levels. This suggests that the effect is likely to be central and also indicates that chronic treatment with AG, regardless of route of administration, increases rather than inhibits glucose-stimulated plasma insulin levels. However, a study giving acute pre-treatment with icv versus acute ip administration of AG to mice prior to an ipGTT would help clarify these unanswered questions. In addition, tissue specific knockout mouse models of ghrelin, Ghsr, or Goat may help to clarify the discrepancy between central and peripheral action of AG.
Pharmacological versus physiological action of AG and dAG

Our data are the first to demonstrate that dAG acts through CNS-GHSR to regulate adiposity and plasma insulin levels. The stomach is the predominant source of circulating dAG. However, dAG was only effective when administered icv whereas peripheral administration at very large pharmacological doses did not elicit changes in fat mass or plasma insulin levels. Furthermore, we used pharmacological doses of dAG that are approximately 1000 times higher than endogenous circulating levels of dAG. Therefore, further clarification is required to address whether this is a pharmacological effect of icv administered dAG or whether this is also a role for endogenously produced dAG. Regardless of pharmacological or a physiological role, our data highlight that dAG has GHSR-dependent biological action in an in vivo system.

Nutritional Status and Age

The current data together with previous reports emphasize that nutritional status and age add another layer of complexity to the ghrelin system’s regulation of energy and glucose metabolism. Both AG and dAG circulating levels fluctuate in between meals which indicates that energy status is an important regulator of ghrelin secretion (Liu, Prudom et al. 2008). Furthermore, we and others find that the orexigenic activity of exogenously administered ghrelin is impaired when animals are fed a HFD indicating excess calories also alter ghrelin function (Briggs, Enriori et al. 2010; Perez-Tilve, Heppner et al. 2011). We find that GOAT plays a role in regulating glucose tolerance only in the fasted state. These data might be explained by recent evidence demonstrating that GHSR heterodimerizes with SST5 receptor, which subsequently
modulates AG action on insulin secretion. Moreover, the heteromer formation is
dependent on nutritional status of the cell. However, whether this mechanism explains
our phenomenon in fasted versus fed Goat-/- requires further investigation. Taken
together, these data provide novel insight into metabolic factors that modulate ghrelin
action and might be useful information when developing and prescribing pharmaceutical
therapies based on ghrelin agonism or antagonism.

*Evolutionary perspective of ghrelin acylation*

These data demonstrate that dAG mimics many of the chronic central actions of AG
including the regulation of lipid storage and glucose uptake into peripheral tissues.
Under prolonged fasting conditions, dAG levels are elevated whereas AG levels remain
low (Liu, Prudom et al. 2008). Our data indicate that the rise in dAG during fasting is
acting in the brain to maximize energy storage as well limit peripheral tissue utilization
of circulating glucose. From an evolutionary standpoint, this action of dAG would be
advantageous for an animal to maintain adequate energy stores and blood glucose
levels that were necessary for survival when calories were scarce. We find that a
distinct action of AG as compared to dAG is the ability of AG to promote hyperphagia.
Our group has reported that lipids from the diet can be used as a substrate for ghrelin
acylation and AG levels are elevated during calorie consumption (Kirchner, Gutierrez et
al. 2009). In earlier times when food availability was scarce and unpredictable, animals
needed to maximize energy consumption when calories were available. Therefore,
when the animal found and consumed food, ghrelin was acylated with incoming lipids.
AG would then signal to the brain to inform the animal that calories are present and that
the animal should consume as many of those calories as possible while they are available. In our current society, the over consumption of calories is unnecessary as food for the majority of people is readily available. Meal feeding may have led to the entrainment of the unique secretory pattern of ghrelin in healthy humans. Under normal meal-feeding conditions, levels of AG and dAG rise immediately before a meal and decline rapidly after meal consumption (Liu, Prudom et al. 2008). The levels of both AG and dAG remain low in between meals. The low inter-meal levels of AG and dAG may have evolved because the brain has been entrained to these scheduled meals and realizes that it does not need to promote maximal calorie storage because the next meal will be available in a few hours. Moreover, it is likely that the low levels of total ghrelin seen in human obesity are a compensatory mechanism to limit food intake and energy storage (Tschop, Weyer et al. 2001).

Targeting GHSR in the CNS as a potential treatment for obesity and T2DM

Our data provide novel insight for the development of obesity and T2DM pharmacotherapies that target the GOAT/Ghrelin/GHSR system. We highlight that ghrelin–GHSR interactions in vivo are complex and cannot be completely predicted by the results of conventional in vitro assays. Our data also highlight a role for dAG acting in the brain to regulate a positive energy balance and suggest that chronic central action of dAG in the brain can lead to the development of peripheral insulin resistance. Based on these findings, therapeutics that target ghrelin acylation (GOAT inhibition) will likely be an ineffective strategy for combating obesity and T2DM as dAG will still be present. Importantly, AG has a wide variety of actions including positive effects on
cardiovascular function (Freeman, Carmo et al. 2013; Soeki, Niki et al. 2013) and prevention of β-cell apoptosis (Granata, Settanni et al. 2007). Therefore, antagonizing ghrelin acylation through the use of GOAT inhibitors may cause negative side effects. The novel interaction of dAG with GHSR that we have identified should be considered in the ongoing efforts towards the development of therapeutics based on dAG action especially those that aim to treat T2DM with dAG analogs (Delhanty, Huisman et al. 2013). Although we find that dAG enhances glucose-stimulated insulin secretion, we also find that dAG impairs glucose clearance from peripheral tissues. Therefore, long-term treatment with dAG analogs may actually worsen rather than alleviate peripheral insulin resistance in T2DM patients.

Further research is required to identify the neuronal populations that mediate AG and dAG action on glucose homeostasis. Identifying these neuronal populations and determining whether they overlap with the neurocircuits that mediate ghrelin’s action on energy homeostasis can help us to understand why T2DM is so highly associated with obesity. In the future, the development of therapeutics that specifically antagonize GHSR activity in these specific neuronal populations will be the most effective strategy for treating obesity an T2DM.

**Overall Conclusions**

AG has a well-established role in regulating food intake, adiposity and GH secretion through CNS mediated mechanisms. These data demonstrate that depending on the length of the FA side-chain acylating ghrelin as well as the route of administration, acyl
ghrelin action can be either enhanced or diminished. Moreover, our data suggest that dAG, which was initially classified as an inactive hormone devoid of GHSR agonism, regulates body weight and adiposity through central GHSR dependent mechanisms. Previous reports demonstrate that AG impairs glucose tolerance by acting directly on pancreatic islets to inhibit insulin secretion. Our data demonstrate that AG as well as dAG act in the CNS to increase glucose-stimulated plasma insulin levels without affecting glucose tolerance. The reason for opposing central and peripheral actions of AG requires clarification, but could indicate why it has been challenging to characterize the endogenous role of the ghrelin system based on global knockout mouse models. Lastly, we identified another layer of complexity to ghrelin’s regulation of glucose metabolism by demonstrating that GOAT action on glucose tolerance is dependent on calorie restriction and age.

Taken together, these data highlight that acylation state as well as metabolic status modulate ghrelin action on energy and glucose metabolism. Employing advanced genetic technology in combination with pharmacology in animals under fed and fasting conditions as well as at different stages of development will be required to gain a deeper understanding of ghrelin physiology.
References


Gauna, C., B. van de Zande, et al. (2007). "Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R)." Mol Cell Endocrinol 274(1-2): 30-34.


Holst, B., E. Brandt, et al. (2005). "Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling." Mol Endocrinol 19(9): 2400-2411.


