Spring 5-1-2015

Characterization of Ghrelin O-acyltransferase Active Site

Leslie Patton
Syracuse University

Characterization of the Human Ghrelin *O*-acyltransferase Active Site

Leslie Michelle Patton

Renée Crown Honor Capstone Project

30 April 2015

Abstract

Ghrelin, first discovered in 1999, is a 28-amino acid peptide hormone involved in the regulation of appetite, insulin secretion and sensitivity, and many neurological effects such as learning, memory, and depression.[1-6] Ghrelin has been identified to have a unique posttranslational octanoylation carried out by the enzyme ghrelin *O*-acyltransferase (GOAT). This distinctive modification is a point of interest in studying GOAT whereby blocking the acylation of the ghrelin could potentially halt the activity of the peptide hormone and provide a means of treating obesity, diabetes, and other diseases affected by ghrelin levels. The duration of my project involved working with a 20-amino acid mimic of the ghrelin peptide with various single residue mutations in the original wild type ghrelin sequence (GSSFLSPEHQRVQQRKESKK). The 20-amino acid ghrelin mimics are fluorescently labeled with a single acrylodan compound, and the activity as well as the inhibitory effects are monitored via reverse phase high performance liquid chromatography.[7] Further studies were done to identify the interactions of ghrelin with GOAT specifically at the N-terminal lysine-5 position of ghrelin. Defining the interactions of ghrelin with the GOAT binding site and octanoyl Co-A substrates would pave the way to design inhibitors and aid in helping diseases related to diabetes, obesity, and neurological illnesses such as Alzheimer's and Parkinson's.

Executive Summary

Ghrelin is a peptide hormone secreted from endocrine cells (cells that release peptides, hormones, steroids, or neuropeptides) in the stomach that plays many roles in the body, among which is stimulating appetite. Studies have shown ghrelin to increase before meals and decrease upon satiation. Similarly, mice injected with additional amounts of ghrelin exhibit increased consumption of food, whereas those with the gene for ghrelin or its receptor (GHSR-1a) knocked out show decreased food consumption.[15] Subsequent research has linked the secretion of ghrelin to maintaining levels of glucose in a state of starvation, learning and memory, insulin secretion, and the production of fat.[1-6, 13]

Ghrelin is a twenty-eight amino acid peptide hormone, that binds and activates the growth hormone secretagogue receptor (GHSR-1a). However, in order for ghrelin to be biologically active and be able to bind to the GHSR-1a receptor, it must be modified with an octanoic acid on a serine at the third position from the N-terminus of ghrelin.[1] This unique modification of ghrelin, required for biological activity, is catalyzed by the enzyme ghrelin O-acyltransferase (GOAT), which attaches an octanoate group ($C_8H_{15}O$) to serine-3 (third amino acid) in the ghrelin sequence GS**S**FLSPEHQRVQQRKESKKPPAKLQPR*.[1] Additionally, ghrelin has shown to be the sole substrate for GOAT, providing further support for the unique nature and function of the ghrelin signaling pathway.[1,11]

As ghrelin is the only known substrate for GOAT, the mechanism of how GOAT catalyzes ghrelin and attaches the octanoyl fatty acid group has been a key point of

* Letters denoting specific amino acids: G, glycine; S, serine, F, phenylalanine; L, leucine; etc.

interest. Potentially blocking ghrelin from binding to GOAT could inhibit the signaling pathways that lead to increased appetite, the production of adipose tissue, and many other effects stimulated by octanoylated ghrelin. To efficiently block ghrelin recognition and modification by GOAT, the interactions between the ghrelin substrate and GOAT must be deciphered.

Prior studies discovered the first five amino acids of the ghrelin sequence elicited reactivity with GOAT—implying those amino acids ('GSSFL') make up the recognition site of ghrelin.[14] The Hougland lab developed a shortened ghrelin peptide consisting of the first six amino acids, rather than the full twenty-eight amino acid wild type sequence, and attached an acrylodan fluorophore at the sixth amino acid in order to allow fluorescence detection and monitoring of acylation of the peptide substrate by GOAT. Optimizing this shortened ghrelin mimic in the reaction with GOAT provided a baseline of reactivity that can be used to compare with subsequent reactions to study the interactions of the amino acids with the GOAT active site. From here, site directed mutagenesis of specific amino acids in the ghrelin sequence could be applied to evaluate the chemical interaction between ghrelin and GOAT involved in ghrelin binding and subsequent acylation.

Previous studies done in the Hougland lab have investigated interactions of the first four amino acids (GSSF). To extend these studies, I explored the characteristics of the fifth amino acid (leucine) in ghrelin binding. Amino acids containing different properties were substituted for leucine at the fifth amino acid position on ghrelin, and the reactivity's of the leucine mutants were compared against the six amino acid substrate. Alanine (smaller sized amino acid), phenylalanine (medium sized amino acid), and

tryptophan (largest amino acid) were substituted to explore the toleration of size GOAT has for ghrelin at this position; whereas glutamate (negatively charged) and lysine (positively charged) were substituted to examine toleration of charge.

Beyond the specific interactions occurring between the peptide hormone and GOAT, we believe a binding pocket exists where ghrelin resides during catalysis. Though the first four amino acids on the N-terminal sequence of ghrelin have been shown to be essential for binding and recognition to GOAT,[11] there is evidence that interactions also occur between downstream portions of ghrelin and GOAT.[16] Determining the length of the binding pocket and which of the twenty-eight amino acids of ghrelin lie within the pocket will aid in identifying downstream interactions and potentially lead to additional targets for the development of GOAT inhibitors. My specific project includes placing the acrylodan fluorophore at different positions on ghrelin in the context of a twenty-amino acid ghrelin mimic. The bulky acrylodan compound can block interactions occurring within the binding pocket of GOAT, while an acrylodan at a position that does not contact GOAT should have a small effect of ghrelin binding. An unreactive acrylodan labeled ghrelin peptide indicates the acrylodan group is blocking binding and that portion of the peptide resides in the active site—while a reactive acrylodan labeled ghrelin mimic suggests the portion of the labeled peptide is not within the group, but instead outside of the binding pocket not interfering in the reaction between ghrelin and GOAT. By "walking out" the placement of the acrylodan compound from the sixth amino acid position to the twentieth amino acid position (the last amino acid), we can probe the approximate length of the ghrelin binding site.

Understanding the precise interactions and characterizing the active site between ghrelin and GOAT is imperative for potentially designing inhibitors to block the binding of ghrelin. An inhibitor that could adequately bind and hinder ghrelin binding to GOAT could be used to lower levels of appetite and food intake, which is highly regulated by the ghrelin-signaling pathway. In addition to treating obesity, blocking the ghrelin pathway could also provide therapeutics to depression, learning and memory, post traumatic stress disorder (PTSD), insulin secretion, and other neurological illnesses and functions associated with the ghrelin signaling pathway.

Table of Contents

Acknowledgements

I would like to thank Dr. James L. Hougland, Joe Darling, the Hougland Research group, Dr. Heather Coleman, the Renée Crown Honors Program, and Syracuse University for supporting and helping through the Capstone process.

Advice to Future Honors Students

Do your Capstone Project on something you enjoy! It’s a great experience to be able to go really in depth about something you find interesting and that you have studied all through out college. That being said, start you Capstone Project early enough too. Begin writing drafts over Christmas break, that way when finals get close you’ll be ready to turn it all in.

Introduction

Secreted from the stomach and pancreas as a 28-amino acid peptide, ghrelin has been shown to regulate appetite, stimulate the release of growth hormone, and control certain neurological processes such as depression and memory, and aid in neuroprotection in diseases like Parkinson's and Alzheimer's.[1-6] Ghrelin has most notably been recognized for its control on appetite as studies have shown it to increase prior to a meal, and decrease after eating, a mechanism transduced through the hypothalamus in the brain.[4] Because of this, finding the precise method of how ghrelin is activated has become a major source of interest in curing obesity. Moreover, increased levels of ghrelin have been linked to high levels of stress, like that in posttraumatic stress disorder (PTSD), linking stress and weight gain.[18] Overall, it is evident ghrelin functions in a vast array of pathways in the body and has become a popular area of study in the past decade.

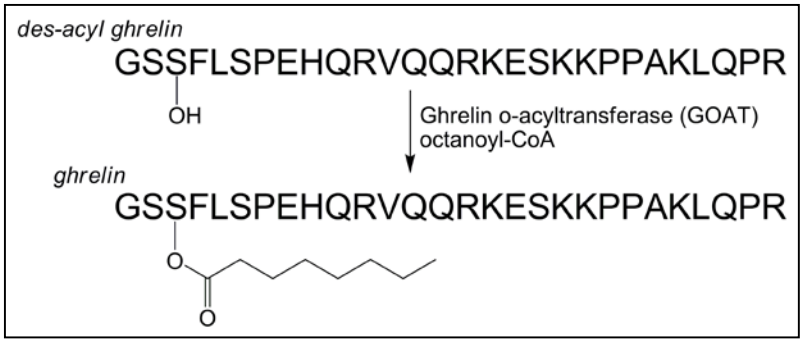


Figure 1. Des-acyl ghrelin precursor requires a unique post-translational modification for ghrelin to become biologically active.

Ghrelin requires a number of post-translational modifications in order to become active, beginning as preproghrelin, a 117-amino acid protein.[8,9] Following translation, preproghrelin is cleaved to a 94-amino acid des-acyl proghrelin where it becomes acylated by a C8 fatty acid group at the serine-3 position of the N-terminus of the peptide. After acylation occurs, acyl-ghrelin is cleaved by a prohormone convertase to its active 28-amino acid form, referred to as ghrelin (**Figure 1**). The body contains both des-

acyl ghrelin as well as the ghrelin, however previous studies have shown that only the acylated form of ghrelin exhibits activity.[2] Upon activation, ghrelin acts as a ligand for the growth hormone receptor GHSR-1a, stimulating the release of growth hormone.[1] Ghrelin is the only known protein to require a serine octanoylation for activity, making it a desirable target for controlling ghrelin signaling.[1,11]

The enzyme responsible for the acylation of ghrelin, and thus responsible for the activity of the peptide hormone, was identified in 2008 as ghrelin *O*-acyltransferase (referred to as GOAT).[†,10,11] GOAT is a member of the MBOAT (membrane-bound O-acyltransferase) enzyme family, however ghrelin is the only known substrate for GOAT. Because ghrelin acylation is required for biological activity, understanding the structure and interactions in the ghrelin-GOAT complex is important to develop inhibitors to block the biological activity of ghrelin. Though the structure of GOAT is unidentified, it is predicted that it is membrane-spanning enzyme of 11 trans-membrane regions bridged by 11 loops domains.[12] Furthermore, the location of conserved residues (Asn307 and His338) among MBOAT family members suggest the active site of human GOAT (hGOAT) lies within the C-terminal region of the enzyme. Though the enzyme poses a novel opportunity for producing therapeutics protecting against many life-threatening illnesses, uncertainty regarding the structure of the active site and the mechanism of ghrelin acylation by hGOAT presents obstacles to creating GOAT inhibitors. Characterization of the specific interactions of the N-terminal region of ghrelin with the C-terminal region of hGOAT will help guide the design of efficient inhibitors of hGOAT.

[†] Abbreviations used: GOAT, ghrelin *O*-acyltransferase; MBOAT, membrane-bound O-acyltransferase; hGOAT, human ghrelin *O*-acyltransferase; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-test of function; MAFP, methyl acarchidonyl fluorophosphonate; TCA, trichloroethane; IC_{50}, half maximal inhibitory concentration

Previous studies done in the Hougland Lab have identified interactions between ghrelin and GOAT by performing amino acid mutations on a peptide substrate that mimics the N-terminal region of ghrelin, with sequence variation of the first four amino acids and monitoring reactivity through a fluorescent-based assay.[7,16] This assay involves mutating the S-6 position to a cysteine (6-mer sequence GSSFLC), creating a shortened ghrelin mimic.[7] The shortened 6-mer peptide is reacted with a fluorescent acrylodan compound to introduce a fluorescent label, which is then monitored via high performance liquid chromatography (HPLC)(**Figure 2**).[7] While the shortened fluorescently labeled peptide ghrelin mimic only shows about 20% product conversion to the acylated form, creating a baseline value of the 6-mer ghrelin substrate reactivity is sufficient for comparing further ghrelin mimics. Prior activity analysis of mutations occurring at residues 1-4 ('GSSF') of ghrelin in accordance with hGOAT has shown interactions imperative for the reactivity of GOAT. The L5 position, ('GSSFL'), was further investigated through site-directed mutagenesis as described below in order to better characterize the interactions occurring within the GOAT binding site with the ghrelin substrate.

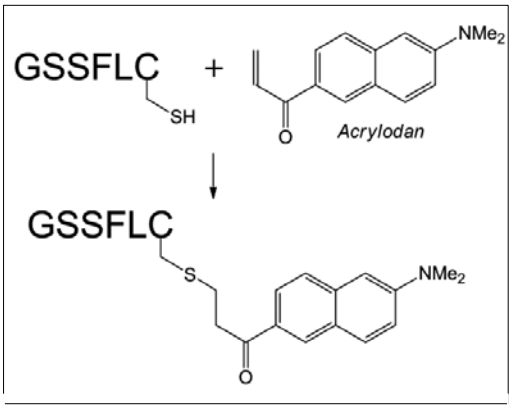


Figure 2. Scheme to labeling ghrelin mimics with the fluorophore compound, acrylodan.

The 6-mer ghrelin mimic substrate is believed to lie in a groove in hGOAT. To test this, my project consists of a 20-mer ghrelin mimic with acrylodan compounds placed at different positions along the peptide sequence. Studies have shown that placing

the acrylodan in the six position ($GSSFLC_{Acdan}$) in the 20-mer substrate has no reactivity with hGOAT because the large aromatic groups of acrylodan are blocking the binding site. Because of this observation, we believe that walking out the acrylodan label on the 20-mer substrate (that is, at the six, ten, fourteen, eighteen, and twenty residue position on ghrelin) could provide insight to the size of the binding pocket ghrelin binds to in hGOAT. Gaining information of the specific contacts ghrelin has with hGOAT could serve as a gateway to designing hGOAT inhibitors.

Materials and Methods

General methods. Octanoyl coenzyme A (octanoyl-CoA) was solubilized to 5 mM in 10 mM Tris-HCl (pH 7.0), and separated into low-adhesion microcentrifuge tubes, and stored at -80 °C. Acrylodan (Anaspec) was solubilized in acetonitrile, with the stock concentration determined by absorbance at 393 nm on dilution into methanol (ε= 18,483 M^{-1} cm^{-1} per manufacturer's data sheet). Unlabeled 20-mer ghrelin peptides mimics were purchased from Sigma Aldrich. 20-mer ghrelin peptide mimics were purified by HPLC, with peptide mass verified by MALDI-TOF. 20-mer ghrelin peptide mimics were solubilized in 50% acetonitrile and stores at -80 °C.

*Expression and enrichment of hGOAT***.** hGOAT was expressed and enriched in insect (Sf9) cell membrane fractions using a previously published procedure.[7]

Peptide substrate fluorescent labeling of Leucine-5 Ghrelin Mutants. A 5,5'-dithiobis-(2-nitrobenzoic acid)

(DTNB) assay was performed to determine free cysteine concentration. The L5 mutant peptide were dissolved in 50% AcCN (200 µl). DTNB solution is made using 0.1 M K_2HPO_4 and 1 mM EDTA. Four mg of DTNB is dissolved in 1 mL of the DTNB solution. To perform the DTNB assay, 50 µl of working solution, 5 µl of L5 mutant peptide, and 945 µl of DTNB solution is mixed and incubated at room temperature for five minutes. Absorbance of the L5 mutant peptides are measured at λ=412 nm (ε=14,150 $cm^{-1}m^{-1}$) and concentration determined using Beers Law. L5 mutant peptide substrates were labeled with an acrylodan fluorophore on a cysteine side chain thiol. Peptide (300 µM) and acrylodan (500 µM) were dissolved in 1 ml of 1:10 50 mM Tris buffer (pH 7.8)/50% acetonitrile, followed by incubation at room temperature in the dark overnight (18 h) with shaking.[17] Acrylodan-labeled peptides were purified by reverse phase HPLC and peptide labeling with acrylodan was verified by MALDI–TOF mass spectrometry (Bruker Autoflex III, SUNY–ESF) using a matrix of 1:1 H_2O: AcCN, 0.1% TFA, and saturated 3-CH(A). Acrylodanylated peptides were solubilized in 50% AcCN and stored at -80° C until use, with peptide concentrations determined by UV absorbance of the cysteine-conjugated acrylodan group at 360 nm in aqueous solution (ε_{360} = 13,300 $M^{-1}cm^{-1}$).

20-mer ghrelin mimics fluorescent labeling. A DTNB assay was performed to determine free cysteine 20-mer ghrelin peptide mimics were labeled with an acrylodan fluorophore on a cysteine side chain thiol. Peptide (300 µM) and acrylodan (500 µM) were dissolved in 50 mM Tris buffer (pH 7.8) and 250 µl 50% acetonitrile. Flick to mix, and vortex for 18 hours at room temperature under foil. The acrylodan-labeled peptides were purified by

reverse phase HPLC and verified by MALDI-TOF mass spectrometry (Bruker Autoflex III, SUNY-ESF) using a matrix of 1:1 H_2O: AcCN, 0.1% TFA, and saturated 3-CH(A). Thoeretical and observed masses are in Table 5. Acrylodanyl labeled 20-mer peptides were dissolved in 50% AcCN and stored in the -80 °C freezer.

hGOAT activity assays and analysis of L5 ghrelin mutants. The membrane fraction of Sf9 cells expressing hGOAT was first thawed on ice, and subsequently broken up through an 18-gauge needle 10 times. Assays utilized 10-50 μg of membrane protein, 2 μM fluorescently labeled peptide substrate, 500 μM octanoyl-CoA, and 50 mM HEPES buffer (pH 7.0), and water to volume up to a total volume of 50 μL. All components were added and mixed by pipetting up and down. The assay began was initiated with the addition of peptide and incubated at room temperature for a total of 1 hour. The reaction was stopped by adding 50 μL of 20% acetic acid in isopropanol. Reverse phase HPLC was used to evaluate the assays through fluorescence detection. Chemstation for LC (Agilent Technologies) was used to integrate peaks of both the peptide and any products, which were presented as a percentage of the total substrate and product fluorescence.

(1) Product Fluorescence= (Maximum Product Fluorescence) * $\left(\frac{[Substrate]}{[Substrate]+Km}\right)$

20-mer Competition Assay to Screen for Inhibition. Membrane protein (10 μL of 2.05 μg/μL stock) was needled ten times using an 18 gauge needle. Fifty μM HEPES, 7.6 μg of membrane fraction, 1 μM MAFP, and water to a total volume of 50 μL was mixed and incubated at room temperature for 10 minutes. To initiate the assay, 100 μM octanoyl Coenzyme A and 7 μM 20-mer peptide mimic was added into the reaction mixture and

incubated for an additional 60 minutes under foil to avoid exposing the fluorescently labeled peptides to light. 8.5 μM ghrelin 6-mer substrate was added to the assay and incubated for another 60 minutes. The assay was stopped with 50 μl 20% acetic acid/isopropanol and activity determined utilizing the following equation:

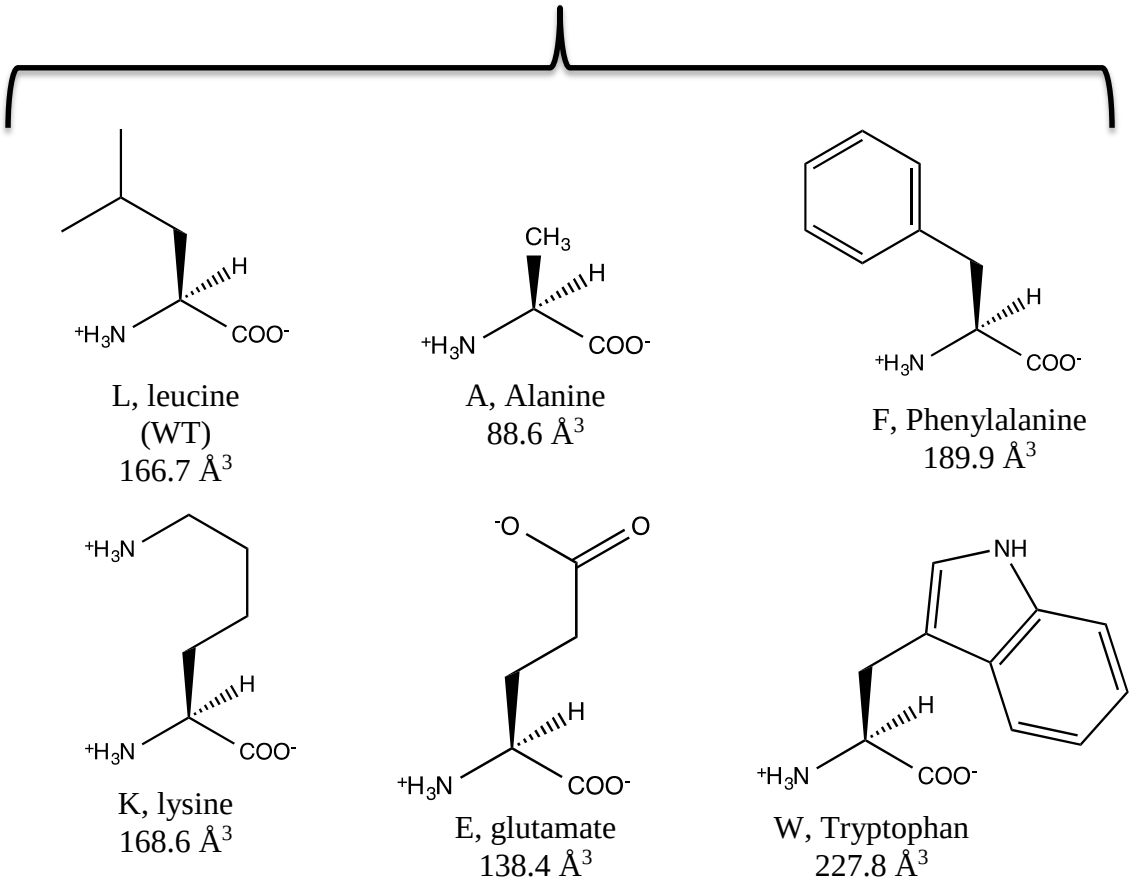
$$(2)\ \%\ \text{activity} = \frac{\%\ \text{peptide octanoylation in presence of inhibitor}}{\%\ \text{peptide octanoylation in absence of inhibitor}}$$

Results

L5 Ghrelin Mimics Series.

Prior experiments in the Hougland lab were done to test the tolerance of hGOAT when the crucial first four N-terminal amino acids ('GSSF') were substituted for other amino acids.[7] To continue to study the characteristics of the binding site at the N-terminus, a series of point mutations were done at the fifth amino acid residue, leucine (**Figure 3a**). These ghrelin mimics were tested for reactivity with hGOAT and the results compared to the reactivity of wild type ghrelin $GSSFLC_{Acdan}$ with hGOAT.

GSSFXC$_{Acdan}$



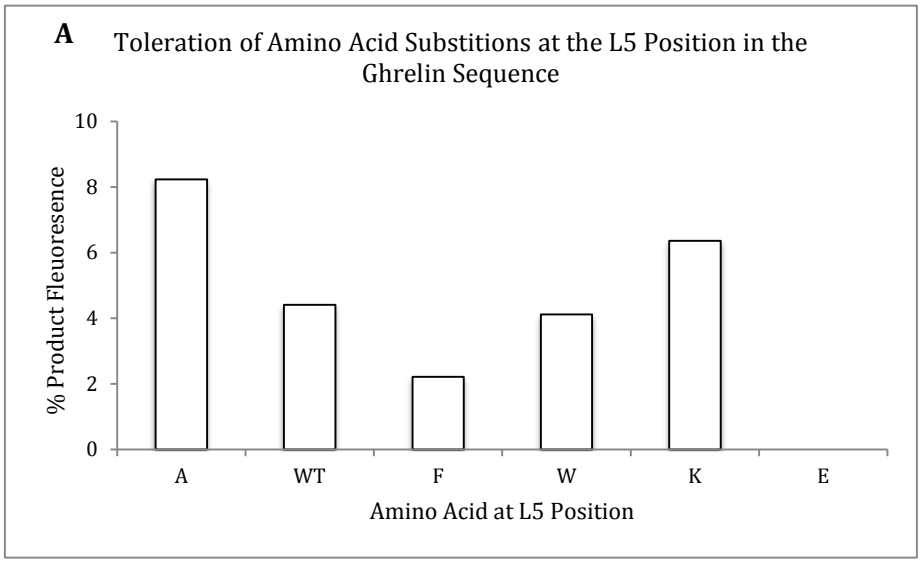
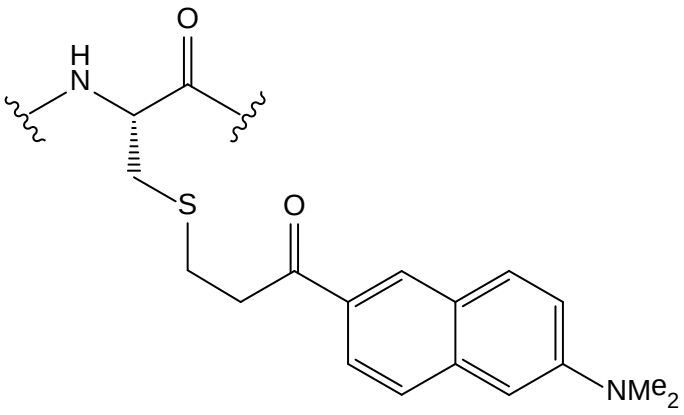
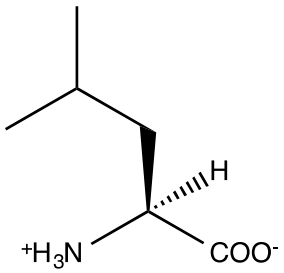


Figure 3a. *Toleration of amino acid substitutions at the L5 position in the wild type (WT) ghrelin sequence*. Amino acids substituted were alanine (A), phenylalanine (F), tryptophan (W), lysine (K), and glutamic acid (E).



Acrylodan Compound
~460 Å^3



Leucine
~166.7 Å^3

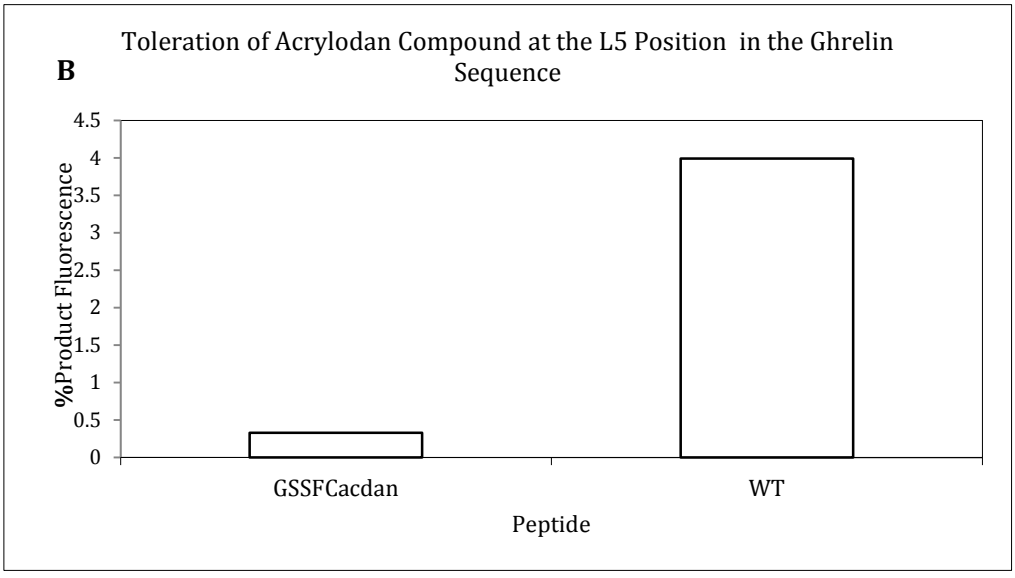


Figure 3b. *Toleration of amino acid substitutions at the L5 position in the wild type (WT) ghrelin sequence*. (b) Reactivity of placing an acrylodan compound at the L5 position.

Placing alanine at the fifth position ('$GSSFAC_{Acdan}$') led to an increase in product fluorescence compared to the wild type $GSSFLC_{Acdan}$ product fluorescence. A rise in the product fluorescence in comparison to the wild type ghrelin substrate indicated the leucine in the wild type sequence is not required for GOAT recognition of ghrelin. Substituting a lysine at the L5 position also led to increased product fluorescence than the wild type, suggesting the hGOAT-binding pocket can tolerate and accept positive charge at this position. Tryptophan substitution led to similar reactivity as observed for the wild type substrate product fluorescence as expected for a hydrophobic residue like leucine. However, the nearly equal product fluorescence of tryptophan, having a volume of 227.8 $Å^3$, to that of leucine, with a surface area on only 166.7 $Å^3$, suggests GOAT is not discriminatory against amino acid size at the fifth residue position. Similarly, phenylalanine produced slightly decreased product fluorescence, however still comparable to the wild type yield with hGOAT. Lastly, placing a negatively charged residue, glutamine, led to a loss of reactivity implying the fifth residue position bears a negative charge.

To further test the fifth residue position, a 5-mer ghrelin mimic peptide was fluorescently labeled with acrylodan ('$GSSFC_{Acdan}$') in order to see how the size and sterics of the fifth amino acid position affects the binding of octanoylation of ghrelin (**Figure 3b**). The large acrylodan almost entirely inhibits the binding of the substrate indicating hGOAT cannot accommodate a compound of that size at that position.

20-mer ghrelin mimic peptide with hGOAT assay.

The specific size and length of the hGOAT binding site remains unknown. Additionally, while interactions between the first five amino acids have been researched,

interactions of ghrelin with hGOAT downstream at positions beyond the 'GSSFLS' sequence are largely undeciphered. Implementing a single cysteine residue mutation at different positions on the wild-type 20-mer ghrelin sequence ('GSSFLSPEHQRVQQRKESKK') was done to create a template for labeling with the fluorescent acrylodan compound. The ghrelin mimics were initially labeled with acrylodan at the cysteine mutation. However, this assay resulted in the double labeling of acrylodan at more than one position on the 20-mer ghrelin mimic sequence. A modified protocol was optimized to result in acrylodan labeling of only the cysteine mutation, with labeling verified MALDI-TOF mass spectrometry (**Figure 5, Table 6**).

Variant	Sequence
WT	GSSFLSPEHQRVQQRKESKK
S6C	GSSFL**C**PEHQRVQQRKESKK
Q10C	GSSFLSPEH**C**RVQQRKESKK
Q14C	GSSFLSPEHQRVQ**C**RKESKK
S18C	GSSFLSPEHQRVQQRKE**C**KK
K20C	GSSFLSPEHQRVQQRKESK**C**

Figure 4. 'WT' indicating the wild type ghrelin sequence. Otherwise, the ghrelin variants are listed by the first letter of the wild type sequence, followed by the point at which the mutation is made, and the last letter indicating the residue substituted.

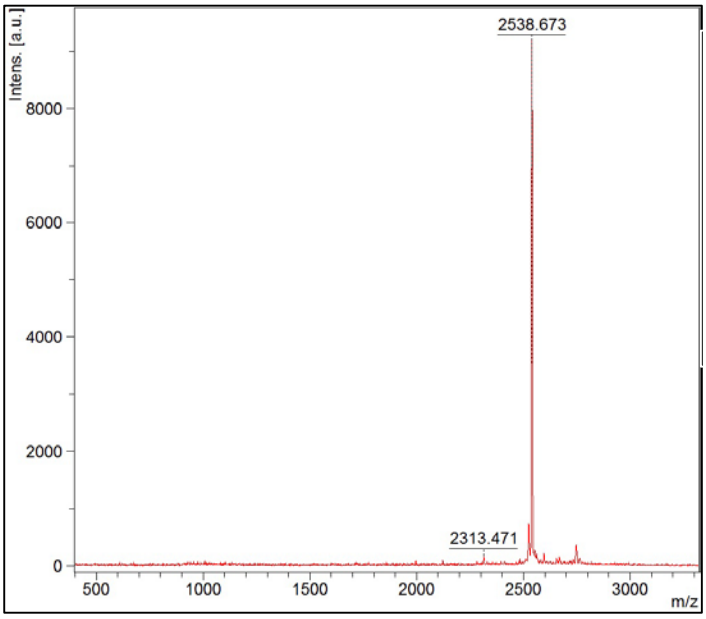


Figure 5. MALDI-TOF on S6C$_{Acdan}$ 20-mer ghrelin mimic. All 20-mer mimics were verified by MALDI-TOF after labeling each peptides with an acrylodan compound to ensure single labeling.

Table 6. MALD-TOF data determining single acrylodan labeling of the 20-mer ghrelin mimics. Theoretical molecular weight (MW) calculated with the addition of 225 g/mol for the acrylodan compound.

Peptide	Sequence	Theoretical MW (g/mol)	Actual MW
Q10S, K20C$_{AcDan}$	GSSFLSPEHSRVQQRKESKC$_{Acdan}$	2525.56	2513.41
Q14C$_{AcDan}$	GSSFLSPEHQRVQC$_{Acdan}$RKESKK	2556.65	2555.38
Q14S, K20C$_{AcDan}$	GSSFLSPEHQRVQSRKESKC$_{Acdan}$	2515.56	2513.45
K20C$_{Acdan}$	GSSFLSPEHQRVQQRKESKC$_{Acdan}$	2556.61	2555.56
Q10C$_{Acdan}$	GSSFLSPEHC$_{Acdan}$RVQQRKESKK	2556.63	2555.25
S18C$_{Acdan}$	GSSFLSPEHQRVQQRKEC$_{Acdan}$KK	2597.69	2594.95
S6C$_{Acdan}$	GSSFLC$_{Acdan}$PEHQKAQQRKESKK	2541.62	2538.67

Introducing the large fluorescent acrylodan compound was done in order to create steric hindrance within the binding pocket to further determine the size and length of the active site. The cysteine mutations (as shown in **Figure 4**) were incorporated systematically throughout the downstream sequence on each 20-mer ghrelin mutant peptide substrate. It was expected the K20C$_{Acdan}$ ghrelin mimic would show reactivity with hGOAT, as it is thought only a portion of the N-terminal substrate resides in the hGOAT active site. Whereas a 20-mer ghrelin mimic with an acrylodan label upstream might not bind due to the large compound blocking the active and longer ghrelin sequence inhibiting the acrylodan compound from altering its position to be able to fit into the active site. Serine point mutations were specifically mutated into the ghrelin sequence on the Q10S, K20C and the Q14S, K20C 20-mer peptides. Implementing these serine mutations ensured that substituting glutamine for the cysteine needed to attach the fluorophore has no effect on ghrelin binding.

To probe the length of the hGOAT active site, an assay was carried out to examine to reactivity of the hGOAT-ghrelin complex using 20-mer ghrelin mimics. Four

components of the hGOAT assay reaction (ultra pure water, 50 μM HEPEs buffer, membrane fraction containing the GOAT enzyme, and 1 μM MAFP) were incubated for 30 minutes. The assay was initiated by the addition of 500 μM octanoyl CoA (the acyl donor) and 1.5 μM of the 20-mer peptide ghrelin mimic. The assay was run for 6 hours and results observed by reverse phase HPLC. The chromatograms from the 20-mer ghrelin and hGOAT assay showed no formation of product. A peptide peak was apparent at 25 minutes, with no product peak that would be expected to appear at a later retention time due to the attachment of a hydrophobic group.

A final study to optimize the assay to be viable with the longer ghrelin substrates was done by omitting a single reaction component during the assay and analyzing reactivity of the assay by reverse phase HPLC. It was found that the preparation of the sample for loading onto the HPLC was pulling the peptide out of solution. This preparation includes adding tricarboxylic acid (TCA) to the sample to precipitate larger proteins from the reaction, and centrifuging the sample for one minute. Because the 20-mer ghrelin mutants could not be observed and analyzed via HPLC, a new route was taken to observe the binding of the 20-mer ghrelin mimic to hGOAT.

Inhibition with 20-mer ghrelin mimics.

As results testing specific activity of the 20-mer substrates were inconclusive, new conditions were developed to examine the 20-mer ghrelin mimics as competitive inhibitors with the 6-mer substrate ($GSSFLC_{Acdan}$) and monitor the reactivity seen in the 6-mer substrates (**Figure 8**).

The S6C 20-mer ghrelin showed very little inhibition, indicating the acrylodan at the sixth amino acid position was almost entirely blocking binding of the 20-mer

substrate, and the 6-mer ghrelin mimic was successfully binding. Likewise, the $Q10C_{Acdan}$ and $Q14C_{Acdan}$ showed nearly 50% inhibition compared to the wild type 6-mer reactivity. The further downstream the acrylodans are placed (i.e. $S18C_{Acdan}$ and $K20C_{Acdan}$) the greater the inhibition seen.

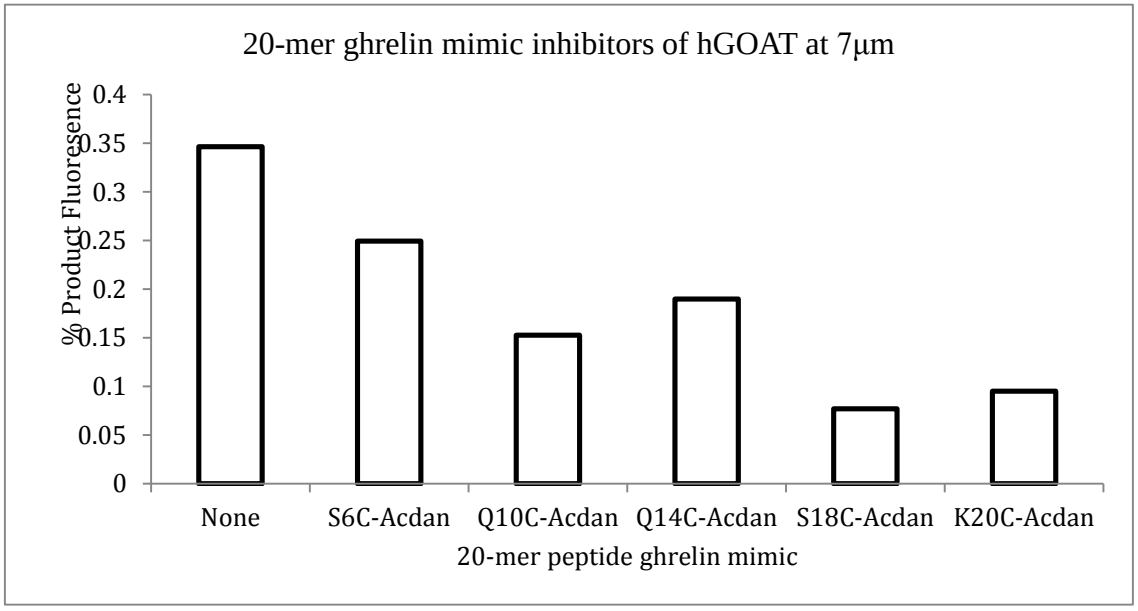


Figure 8. 20-mer ghrelin mimic inhibitors at 7µm. Inhibition seen with the S18C-Acdan and K20C-Acdan ghrelin mutants, which could indicate those amino acids fall outside of a proposed ghrelin binding pocket.

Discussion

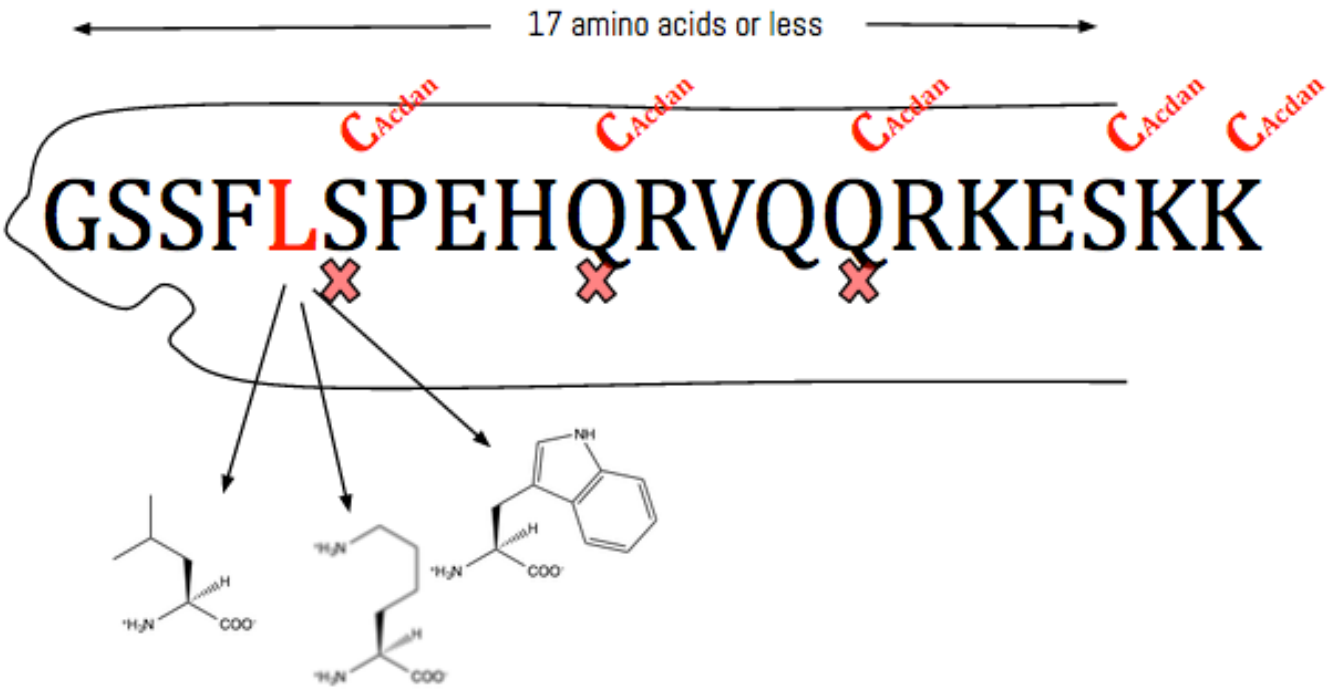
Characterization of the ghrelin *O*-acyltransferase binding site through inhibition studies of 20-mer ghrelin mimics led to a better estimation of the length of the binding site. It was found that our previous methods to monitor activity based on an assay of ghrelin with hGOAT could not be sued to visualize the reactivity of the longer ghrelin substrate. As a means to monitor the binding and acylation of ghrelin, an inhibition assay was carried out with 20-mer ghrelin mimics in competition with a 6-mer ghrelin substrate

to bind with hGOAT. A concern when using the acrylodan label is ensuring the cysteine mutation required for acrylodan labeling does not alter ghrelin binding with hGOAT. In attempt to assess the implications of the cysteine mutation and taking away of glutamine residues to attach a fluorophore, serine point mutations were created on ghrelin at the Q10 and Q14 position with the acrylodan label at the 20th residue in both cases. The data obtained suggests a binding pocket exists on the enzyme where ghrelin binds to and is acylated, as placing the acrylodan label at different positions on the sequence results in vastly different reactivity. From the 20-mer ghrelin mutants inhibition assay, the binding site is between 14-17 amino acids in lengths, as inhibition of hGOAT greatly increases with the S18C-Acdan and K20C-Acdan substrates.

In addition to better determining the size of the binding site, it can be concluded the leucine at the fifth residue from the N-terminal sequence is not essential for ghrelin to be recognized by hGOAT, which is not the case for the first four amino acids before it. Moreover, hGOAT is not discriminatory against size with tryptophan resulting in no decrease in ghrelin binding. Interestingly, there is a drop in reactivity with phenylalanine placed at the fifth position. Because there is no drop in reactivity with the larger tryptophan mutant (L5W), it can be assumed there are other properties interacting with the active site at that position. The increased reactivity with the lysine mutant (L5K) and similar reactivity to wild type ghrelin with tryptophan (L5W) gives insight to possible hydrogen bonding occurring at that residue position on ghrelin with the hGOAT enzyme. Further, the complete rejection of the glutamate mutant (L5E) suggests a negative charge resides on the enzyme near that position. From this data, a rough model of the hGOAT active site was developed (**Figure 9**).

Defining the mechanism and layout of the ghrelin substrate binding with hGOAT is an important step to designing inhibitors that can block the binding site and subsequently block the ghrelin-signaling pathway. Due to the difficulties of analyzing the integral membrane enzyme hGOAT by high-resolution methods, probing the ghrelin substrate with different mutations and monitoring the reactivity with hGOAT based on the formation of the acylated product is a sufficient way to depict the ghrelin-GOAT interactions. By systematically identifying specific characteristics of the ghrelin-GOAT complex, synthesis of inhibitors that take advantage of the hGOAT binding properties of ghrelin can be created. Blocking the ghrelin signaling pathway could pave a way for potential therapeutics curing diseases such as post-traumatic stress disorder[18], Prader-Willi Syndrome, diabetes, obesity, and many more.[19-21]

Figure 9. *Proposed binding site of ghrelin-hGOAT complex*.



References

1. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach, *Nature 402* , 656-660.
2. M. Kojima, K. Kangawa, Structure and function of ghrelin, Results Probl. Cell Differ. 46 (2008) 89-115.
3. S. Diano, S.A. Farr, S.C. Benoit, E.C. McNay, I. da Silva, B. Horvath, F.S. Gaskin, N. Nonaka, L.B. Jaeger, W.A. Banks, J.E. Morley, S. Pinto, R.S. Sherwin, L. Xu, K.A. Yamada, M.W. Sleeman, M.H. Tschop, T.L. Horvath, Ghrelin controls hippocampal spine synapse density and memory performance, Nat. Neuroci. 9 (2006) 381-388.
4. M. Lutter, I. Sakata, S. Osborne-Lawrence, S.A. Rovinsky, J.G. Anderson, S. Jung, S. Birnbaum, M. Yanagisawa, J.K. Elmquist, E.J. Nestler, J.M. Zigman, The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress, Nat. Neurosci. 11 (2008) 752-753.
5. Z.B. Andrews, D. Erion, R. Beiler, Z.W. Liu, A. Abizaid, J. Zigman, J.D. Elsworth, J.M. Savitt, R. DiMarchi, M. Tschoep, R.H. Roth, X.B. Gao, T.L. Horvath, Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism, J. Neurosci. 29 (2009) 14057-14065.
6. M.D. Gahete, J. Corboda-Chacon, R.D. Kineman, R.M. Luque, J.P. Castano, Role of ghrelin system in neuroprotection and cognitive funtions: implications in Alzheimer's disease, Peptides 32 (2011) 2225-2228.
7. J.E. Darling, E.P. Prybolsky, M. Sieburg, J.L. Hougland, A fluorescent peptide substrate facilitate investigation of ghrelin recognition and acylation by ghrelin O-acyltransferase, Anal. Biochem. 437 (2013) 68-76.
8. A. Zhou, G. Webb, X. Zhu, D.F. Steiner, Proteolytic processing in the secretory pathway, J. Biol. Chem. 274 (1999) 20745-20748.
9. X. Zhu, Y. Cao, K. Voogd, D.F. Steiner, On the processing of proghrelin to ghrelin, J. Biol. Chem. 281 (2006) 38867-38870.
10. J.A. Gutierrez, P.J. Solenberg, D.R. Perkins, J.A. Willency, M.D. Knierman, Z. Jin, D.R. Witcher, S. Luo, J.E. Hale, Ghrelin octanoylation mediated by an orphan lipid transferase, Proc. Natl. Acad. Sci. USA 105 (2008) 6320-6325.
11. Yang, J., Brown, M.S., Liang, G., Grishin, N.V., Goldstien, J.L., (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone, *Cell 132,* 387-396.
12. Taylor, M. S., Ruch, T. R., Hsiao, P. Y., Hwang, Y., Zhang, P., Dai, L., Huang, C. R., Berndsen, C. E., Kim, M. S., Pandey, A., Wolberger, C., Marmorstein, R., Machamer, C., Boeke, J. D., and Cole, P. A. (2013) Architectural organization of the metabolic regulatory enzyme ghrelin o-acyltransferase, *J Biol Chem 288*, 32211-32228.
13. Goldstien, J.L., Zhao, T.J., Li, R.L., Sherbet, D.P., Liang G., Brown, M.S., (2011) Surviving Starvation: essential role in ghrelin-growth hormone axis, *Cold Spring Harb Symp Quant Biol 76,* 121-127.

14. Yang, J., Zhao, T.J., Goldstien, J.L., Brown, M.S., (2008) Inhibition of ghrelin *O*-acyltransferase (GOAT) by octanoylated pentapeptides, *Biochemistry 31*, 10750-10755.
15. Zigman, J.M., Nakano, Y., Coppari, R., Balthasar, N., Marcus, J.N., Charlotte, L.E., Jones, J.E., Deysher, A.E., Waxman, A.R., White, R.D., Williams, T.D., Lachey, J.L., Seeley, R.J., Lowell, B.B., Elmquist, J.K., (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity, *J Clin Invest 12*, 3564-3572.
16. Darling, J.D., Zhao, F., Loftus, R.J., Patton, L.M., Gibbs, R.A., Hougland, J.L., (2015) Structure-activity analysis of human ghrelin O-acyltransferase reveals chemical determinants of ghrelin selectivity and acyl group recognition, *Biochemistry 54*, 1100-1110.
17. M.A. Tricerri, A.K. Behling Agree, S.A. Sanchez, A. Jonas, (2000) Characterization of apolipoprotein A–I structure using a cysteine-specific fluorescence probe, *Biochemistry 39*, 14682–14691.
18. Meye, R.M., Burgoes-Robles, A., Correia, S.S., Goosens, K.A., A ghrelingrowth hormone axis drives stress-induced vulnerability to enhance fear, *Mol. Psych*. **2014**, *19*, 1284-1294.
19. Heppner, K. M., Tong, J., Kirchner, H., Nass, R., and Tschop, M. H. (2011) The ghrelin o-acyltransferase-ghrelin system: A novel regulator of glucose metabolism, *Curr Opin Endocrinol Diabetes Obes 18*, 50-55.
20. Hillman, J. B., Tong, J., and Tschop, M. (2011) Ghrelin biology and its role in weight-related disorders, *Discov Med 11*, 521-528.
21. Yi, C. X., Heppner, K., and Tschop, M. H. (2011) Ghrelin in eating disorders, *Mol Cell Endocrinol 340*, 29-34.