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Trends Endocrinol Metab. Author manuscript; available in PMC 2021 February 01.

Published in final edited form as:

Trends Endocrinol Metab. 2020 February ; 31(2): 107–117. doi:10.1016/j.tem.2019.09.006.

Ghrelin signaling: GOAT and GHS-R1a take a LEAP in complexity

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Abstract

Ghrelin and the growth hormone secretagogue receptor 1a (GHS-R1a) are important targets for disorders related to energy balance and metabolic regulation. Pharmacological control of ghrelin signaling is a promising avenue to address health issues involving appetite, weight gain, obesity, and related metabolic disorders, and may be an option for patients suffering from wasting conditions like cachexia. In this review, we summarize recent developments in the biochemistry of ghrelin and GHS-R1a signaling. These include unravelling the enzymatic transformations that generate active ghrelin and the discovery of multiple proteins that interact with ghrelin and GHS-R1a to regulate signaling. Furthermore, we propose that harnessing these processes will lead to highly selective treatments to address obesity, diabetes, and other metabolism-linked disorders.

Keywords

Ghrelin; ghrelin O-acyltransferase; GHS-R1a; LEAP2; GPCR dimers; energy balance; obesity; diabetes

Introduction

Two decades ago, the peptide hormone ghrelin was discovered as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a) (see Glossary) [1]. In contrast to most other gut derived peptides, ghrelin increased caloric intake, decreased energy expenditure, and promoted fat deposition [2]. For these reasons, ghrelin has since been a target for the development of pharmaceutical interventions aimed at curbing obesity and metabolic disorders like cachexia, anorexia nervosa, obesity, and type II diabetes. In the quest to generate ghrelin based therapeutic interventions for these conditions, we have come to a better understanding of how mature ghrelin is generated, how it binds to the GHS-R1a, and the multiple signaling cascades that are stimulated by ghrelin when bound to the GHS-R1a. In this review we focus on research data showing the uniqueness of the processes that

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result in the formation of the active form of ghrelin and the intracellular signaling events that occur following stimulation of the GHS-R1a by mature ghrelin, or by compounds that act as agonists, inverse agonists, or antagonists to this receptor, highlighting the potential for these processes as therapeutic targets.

Ghrelin Acylation By GOAT

At ghrelin's discovery, a unique posttranslational modification - an octanoylated serine - was identified within the highly conserved N-terminal sequence of ghrelin [1]. This acyl modification is essential for binding and activation of the GHS-R1a receptor [3], and recent modeling studies have unveiled interactions between ghrelin and its receptor that lead to activation of downstream signaling [4, 5]. In addition to full length 28 amino acid form of ghrelin, an exon-deleted splice variant of ghrelin has been identified in multiple vertebrate species that yields a 13 amino acid "minighrelin" [6]. When octanoylated, minighrelin exhibits similar biological activity to ghrelin at the cell and organism level suggesting that ghrelin splice variants may play roles in ghrelin signaling.

Octanoylation of the ghrelin precursor proghrelin is catalyzed by ghrelin *O*-acyltransferase (GOAT), a member of the membrane bound *O*-acyltransferase (MBOAT) enzyme superfamily (Figure 1) [7–15]. The structure of GOAT has not been determined beyond a membrane topology model [16], leaving the enzyme active site and substrate binding sites unknown. Lack of structural information remains a key challenge in studies of MBOAT family members, although a recently reported crystal structure of a bacterial MBOAT D-alanyl transferase DltB and a biochemically-validated computational model of human GOAT provide valuable insights into MBOAT catalysis [17, 18]. Functional and biochemical studies have greatly expanded our understanding of how GOAT recognizes its ghrelin and octanoyl-CoA substrates [19–23], and intriguingly these studies indicate ghrelin is a unique substrate for GOAT within the human proteome [20].

Following secretion into the bloodstream, acyl ghrelin has a limited lifetime before it is converted to desacyl ghrelin due to esterase-catalyzed diacylation of the serine octanoyl ester (Figure 1) [24]. Multiple proteins in human serum have demonstrated ghrelin esterase activity including platelet activating factor (PAF), paraoxanase (PON), carboxypeptidase, butylcholinesterase, carboxylesterases, APT1, and alpha 2-macroglobulin [24–28]. Among these esterases, several recent studies support butyrlcholinesterase (BChE) as an important contributor to acyl ghrelin deacylation in humans [29–32]. Fully defining the deacylation aspects of the ghrelin signaling pathway in circulation remains an important challenge in understanding the regulation of ghrelin signaling.

GOAT Inhibitors Enable Pharmacological Control of Ghrelin Signaling

With ghrelin requiring octanoylation to bind the GHS-R1a receptor, inhibiting GOAT-catalyzed ghrelin octanoylation presents an attractive option for modulating ghrelin signaling. Towards this goal, several classes of GOAT inhibitors have been described in the scientific and patent literature. Product-mimetic inhibitors with the ester linkage of octanoylated ghrelin replaced with an amide linkage remain among the most potent

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inhibitors known against GOAT, with an eight-carbon acyl chain providing the tightest binding [20, 23]. Replacement of the amide linked lipid chain with a triazole-linked phenylalkyl group yielded a potentially more biostable ghrelin mimetic inhibitor with submicromolar potency [33]. A recent study of a substrate-mimetic ghrelin analog containing a diaminopropanoic acid (Dap) group in place of the acylation site serine demonstrated unexpected potency against GOAT, with this substitution leading to a >400-fold increase in binding affinity to GOAT [22]. Without needing an acyl group for potency against GOAT, these substrate-mimetic peptides provide an opportunity for GOAT inhibitors with reduced potential for GHS-R1a receptor agonism [3].

Studies employing GO-CoA-Tat, a bisubstrate analog inhibitor of GOAT, remain the strongest support for GOAT inhibition as a therapeutic avenue targeting ghrelin-dependent physiological processes [34–36]. GO-CoA-Tat combines aspects of ghrelin and octanoyl-CoA in a peptide-based molecule which inhibits ghrelin acylation at micromolar or lower concentrations in enzyme- and cell-based assays and decreases serum acyl ghrelin levels in animal studies [36]. GO-CoA-Tat treatment also reduced weight gain in mice fed a medium-chain triglyceride-rich high-fat diet and improved insulin response to a glucose challenge.

While there have been very few known small molecule “drug-like” GOAT inhibitors identified, studies in the last three years have begun to disclose non-peptide based molecules with potency against GOAT. In a report from our research group, a class of molecules derived from synthetic triterpenoids was found to inhibit the human GOAT (hGOAT) ortholog [37]. Two derivatives of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) act as hGOAT inhibitors with low micromolar IC_{50} values in an microsomal hGOAT activity assay [19, 38]. These molecules act as covalent reversible inhibitors of hGOAT, suggesting either the involvement of a cysteine in the hGOAT catalytic mechanism and/or as part of an inhibitor binding site. Surprisingly, mouse GOAT is less sensitive to these cysteine-modifying inhibitors, indicating an important distinction between these two closely related enzyme orthologs. CDDO derivatives have been studied as potential therapeutics for inflammation and oxidative stress in multiple cell signaling pathways including the Nrf2 and NF-κB pathways [39]. Treatment with CDDO derivatives produces side effects such as weight loss, reduced insulin resistance, and improved glucose tolerance in animal and human studies [40–43]. These side effects are consistent with reduced ghrelin signaling resulting from GOAT inhibition by CDDO derivatives, supporting future studies of the physiological impact of CDDO derivatives and similar molecules on ghrelin signaling.

Small-molecule GOAT inhibitors have also been reported by industrial research groups. Two published patents assigned to Eli Lilly report substituted piperidyl-ethyl-pyrimidine derived GOAT inhibitors with potency in enzyme-, cell-, and animal-based studies. The lead compound in this class was discovered using a high-throughput ELISA-based GOAT activity screening assay, and optimized inhibitors were reported to inhibit GOAT in the mid-nanomolar range in *in vitro* GOAT activity assays [44, 45]. In a patent assigned to Takeda Pharmaceuticals, a number of small molecules featuring multiple aromatic rings are reported to potently inhibit GOAT activity using an ELISA-based high throughput assay for detection of acyl ghrelin [46]. In a subsequent publication, two of these Takeda inhibitors are discussed including their behavior as acyl donor-competitive inhibitors [47]. Compounds

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from both the Eli Lilly and Takeda work have been licensed for development towards preclinical studies and clinical trials.

Ghrelin Reacylation By Cell Surface-Exposed GOAT: A New Limb On The Ghrelin Signaling Pathway?

The pathway for secreted ghrelin maturation and acylation has been well established, but two recent studies suggest the potential for a new limb of the ghrelin signaling pathway involving ghrelin reacylation at the cellular site of signaling [48, 49]. In mouse bone marrow, both unacylated and acylated ghrelin promoted adipogenesis in the presence of GOAT. However, the adipogenic effect of unacylated ghrelin was completely absent in GOAT knockout mice, indicating that GOAT-catalyzed octanoylation of unacylated ghrelin and subsequent signaling through GHS-R1a is required to stimulate adipogenesis. Immunogold labeling detected GOAT in intracellular lipid-trafficking vesicles as well as the plasma membrane in bone marrow adipocytes [48], providing evidence of GOAT localization beyond the endoplasmic reticulum (ER) [16]. In another study of hippocampal neurons, fluorescently labeled ghrelin and desacyl ghrelin both bound to cell surface receptors although desacyl ghrelin does not bind the GHS-R1a receptor [3, 49]. GOAT inhibitor treatment blocked desacyl ghrelin binding, consistent with desacyl ghrelin octanoylation by surface-exposed GOAT enabling binding to the GHS-R1a receptor. Local reacylation of desacyl ghrelin would enable cells and tissues expressing both GOAT and the GHS-R1a receptor to integrate the total ghrelin concentration (ghrelin + desacyl ghrelin) in circulation, providing a new and unanticipated dimension to ghrelin signaling (Figure 1).

GHS-R1a Signaling Mechanisms

Once secreted, acyl-ghrelin binds to GHS-R1a in multiple central and peripheral targets to modulate energy homeostasis, reproduction, cognition, reward and emotion [50, 51]. These targets include most peripheral tissues and endocrine glands like the pituitary gland, pancreas, liver, adrenal glands, reproductive tissues, immune cells, skeletal and muscle cells, and brain [52, 53]. It is not clear whether acyl-ghrelin from the periphery can actually reach the brain to reach all regions expressing GHS-R1a. Peripheral ghrelin administration only stimulates brain regions like the ARC or the AP that have a more permeable blood brain barrier, or the NTS which might be stimulated indirectly by ghrelin sensitive vagal afferents [2, 54]. Recent papers suggest that ghrelin enters the brain through blood vessels in the median eminence, where it gets transported to the ventricular system to be transported to multiple brain regions, but more evidence is needed to support this contention [55, 56]. In addition, there is some evidence that splice variants of the ghrelin gene that are also modified by GOAT are produced in the brain, are increased following a shortage in energy fuels, and stimulate the GHS-R1a [57]. In contrast, des-acyl ghrelin does not bind to the GHS-R1a, and while this form of the ghrelin peptide seems to have some physiological and behavioral effects its mechanism(s) of action remain to be elucidated [58, 59].

GHS-R1a is a G-coupled protein receptor that recruits a variety of cell signaling cascades, some of which are associated with the classic physiological and behavioral effects of ghrelin (Figure 2). GHS-R1a is most commonly associated with $G\alpha_{q/11}$ signaling including the

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activation of the phospholipase C pathway (PLC) and consequently inositol phosphate accumulation and signaling, leading to the mobilization of intracellular calcium (Ca^{+}) stores and downstream activation of the cyclic AMP response element binding protein (CREB) transcription factor [50, 60, 61]. While the GHS-R1a is also coupled to several other g-coupled protein complexes, hypothalamic $G\alpha_{q/11}$ signaling in NPY/AGRP neurons is critical for the full orexigenic effects of ghrelin, as mice with mutations to $G\alpha_{q/11}$ in NPY/AGRP neurons do not show a full feeding response to peripheral injections of ghrelin, whereas mice with deletion of $G\alpha_{13/12}$ in NPY/AGRP neurons continue to show ghrelin induced feeding responses [62].

Ghrelin stimulation of the $G\alpha_{q/11}$ pathway also impacts signaling cascades implicated in cell metabolic processes. Ghrelin-stimulated Calcium calmodulin Kinase (CaCMK) activation facilitates the phosphorylation of adenosine monophosphate-activated protein kinase (pAMPK), and within the hypothalamus this intracellular event produces enzymatic changes and lead to alterations in mitochondrial function and energy production [61]. The combination of these events is associated with increased use of fatty acids as an energy substrate in the mitochondria, increased free radical production, increased expression of uncoupling proteins (particularly uncoupling protein 2, UCP2), stimulation of mitochondria biogenesis, increased expression of NPY/AGRP and ultimately increased feeding [61, 63]. Increases in UCP2 following GHS-R1a stimulation have also been associated with neuroprotection, potentially setting GHS-R1a signaling as an important mechanism underlying the neuroprotective effects of caloric restriction [63, 64].

Finally, ghrelin stimulation of GHS-R1a $G\alpha_{q/11}$ protein complexes may also trigger endogenous cannabinoid release that could further enhance pAMPK activated pathways [65]. Activation of PLC and acyl-CoA leads to increased synthesis of diacylglycerol (DAG), a lipid that acts as a second messenger and, in the presence of diacylglycerol lipase (DAGL), is converted into the endocannabinoid 2-arachidonoylglycerol (2-AG), an endocannabinoid known to increase feeding through the stimulation of cannabinoid receptor 1 (CB1) [66, 67]. Interestingly, orexigenic responses to ghrelin require the presence of CB-1 [68–70]. Similarly, feeding responses produced by cannabinoid agonists are attenuated in GHS-R1a KO mice [69–71]. These data suggest that GHS-R1a signaling could recruit the cannabinoid system by increasing 2-AG synthesis and release, and by the synergy between GHS-R1a and CB-1 to increase levels of pAMPK [71]. This interaction has been suggested to occur in regions like the hypothalamus and VTA to influence feeding and reward seeking behaviors and requires more vigorous research [69, 70, 72].

Early studies on the *In Vitro* biochemical properties of the GHS-R1a showed that this receptor was constitutively active as determined by increased PLC activity, IP accumulation and intracellular calcium release in the absence of its ligand [73]. Interestingly, a missense mutation that decreases GHS-R1a constitutive activity, leads to a deficit in growth hormone secretion and subsequent short stature, an effect corrected with exogenous growth hormone treatment [74]. In mice, intracerebroventricular (icv) treatment with inverse agonists decreases body weight and caloric intake after a fast showing that GHS-R1a constitutive activity in the brain is an important modulator of energy balance [75]. At the molecular level, GHS-R1a constitutive activity has been related the trafficking of voltage gated calcium

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channels through mechanisms independent of $G\alpha_{q/11}$ signaling. In the hypothalamus and hippocampus, GHS-R1a constitutive activity has been linked to a reduction in Ca_v2 calcium channels resulting in a reduction of GABA release [76, 77]. Thus, decreased constitutive activity via inverse agonists may increase inhibitory tone and ultimately alter the overall function in these regions.

The activation of the GHS-R1a also involves increased activity of the β-arrestin pathway, a pathway linked to additional kinase activity and also linked to receptor internalization [78]. Mutations to the GHS-R1a that result in a truncated C-terminal also result in reduced ligand-bound GHS-R1a internalization and β-arrestin recruitment [79]. Interestingly, this mutation also enhances ghrelin or ghrelin agonist induced Ca+ mobilization produced by increased PLC activity, and increased serum response element (SRE) transcriptional activity, reflecting increased ghrelin-induced activation of the ERK pathway [79]. Collectively these data suggest that GHS-R1a recruitment of the β-arrestin pathway is required for receptor internalization, and this process may be important for regulating GHS-R1a responses to ghrelin and other ligands. In fact, rats with an *N*-ethyl-*N*-nitrosourea (ENU)-mediated point mutation that truncates the GHS-R1a sequence and results in a receptor protein lacking the C-terminal appear to be more sensitive to the tonic effects of ghrelin possibly though decreased ability of the GHS-R1a to be internalized [79, 80]. These GHS-R1a mutants, however, show attenuated feeding responses after an overnight fast and in response to an acute peripheral injection of ghrelin [81]. Moreover, these rats also fail to show the effects of ghrelin on gastric motility [80, 81]. It is therefore possible that an intact C terminal in the GHS-R1a is required for β-arrestin induced internalization and regulation of receptor signaling, but it may also be required for ghrelin induced effects on feeding and gastric motility.

The dissection of the specific signaling pathways activated by GHS-R1a constitutive activity and ghrelin-induced GHS-R1a stimulation and their roles on behavioral responses is critical for the development of ghrelin or GHS-R1a targeted pharmacological treatments. This dissection would also allow for better understanding of the pathways and signaling partners that are activated by ghrelin analogs, inverse agonists and antagonists currently used in pre-clinical and clinical approaches. Indeed, there are a number of compounds currently being used as GHS-R1a agonists, antagonists or inverse agonists that often have unexplained physiological and/or behavioral responses. For instance, the GHS-R1a antagonist BIM28163, while inhibiting ghrelin induced growth hormone secretion, food intake and Fos expression in the ARC, also increases body weight and Fos expression in the DMH [82]. While it is possible that these divergent effects are mediated by the action of ghrelin and/or BIM28163 on a receptor other than the GHS-R1a, it is more likely that the agonist effects of BIM28163 on the DMH (but not the ARC) are mediated by biased GHS-R1a signaling. Indeed, there is convincing evidence showing that other compounds described as GHS-R1a antagonists can act as agonists when used in the absence of ghrelin or its agonists and that they can bias intracellular GHS-R1a signaling. For instance, in the presence of ghrelin or ghrelin analogs, JMV2959 attenuates ghrelin induced intracellular Ca^+ and pERK as would be expected of an antagonist [62]. However, JMV2959 increases intracellular Ca^+ when used in the absence of a GHS-R1a agonist, and also prevents GHS-R1a internalization [62]. It is therefore possible that JMV2959 prevents ligand induced activation of GHS-R1A $G\alpha_{q/11}$

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signaling, but by reducing GHS-R1a internalization JMV2959 on its own produces a weak agonist effect (Figure 3) [62, 83]. In contrast, this effect is not seen in D-Lys GHRP6, another compound commonly used as a GHS-R1a receptor antagonist [83].

Biased signaling of the GHS-R1a is also due in part to the dimerization properties of this receptor. During the past decade, a number of studies have identified that the GHS-R1a forms homo and heterodimers, and that these dimers regulate GHS-R1a activity and function [84]. For instance, the GHS-R1a dimerizes with the GHS-R1a1b, a truncated splice variant of the GHS-R1a that is not responsive to ghrelin or ghrelin agonists. GHS-R1a/GHS-R1a1b dimerization results in GHS-R1a internalization and decreased ligand binding *In Vitro* [84]. While there is no *In Vivo* evidence of GHS-R1a/GHS-R1a1b dimerization, there is strong evidence that the GHS-R1a forms heterodimers with other G-coupled protein receptors and these dimers are important for a number of processes in the absence of ghrelin [85]. Dopamine D1 and D2 (DRD1 and DRD2) receptors are co-localized with GHS-R1a in a number of brain regions that include the ARC, hippocampus and striatum. Within the ARC, the GHS-R1a colocalizes with DRD2, and treatment with DRD2 agonists like cabergoline increases the formation of DRD2/GHS-R1a heterodimers *In Vivo* and *In Vitro*. The formation of these dimers is independent of ghrelin binding to the GHS-R1a, and biases DRD2 signaling resulting in increased $G\alpha_{q/11}$ signaling, and ultimately mobilization of intracellular Ca^{+} stores [86]. Importantly, this effect requires the presence of the GHS-R1a, as DRD2 stimulation does not increase $G\alpha_{q/11}$ signaling in the absence of GHS-R1a. *In Vivo*, treatment with the dopamine agonist cabergoline decreases food intake, an effect that requires a functional GHS-R1a [86]. The GHS-R1a also forms dimers with DRD1 receptors *In* Vitro and *In Vivo* in response to DRD1 agonists or GHS-R1A agonists [87, 88]. In the hippocampus (but not in the striatum or hypothalamus), the GHS-R1A forms heterodimers with DRD1 and activation of DRD1 results in the formation of DRD1/GHS-R1a heterodimers that bias the receptor complex to signal through the $G\alpha_{q/11}$ pathway, and disruption of these heterodimers has been proposed as a mechanism for the development of Alzheimer's disease [87, 89]. This sequence of events is associated with synaptic plasticity in hippocampal slices and with improved performance in learning and memory tasks [87]. Interestingly, the ability of DRD1 agonists to enhance hippocampal plasticity and performance in these tasks is dependent on the presence DRD1/GHS-R1a dimers, given that GHS-R1a KO mice do not show DRD1 agonist-mediated increases in hippocampal plasticity or better performance in memory tasks [87]. These data suggest that GHS-R1A/dopamine receptor dimers have a functional role independent of ghrelin, and that specific dimerization can occur in different brain regions to bias signaling, and alter behaviors associated with feeding, cognition and emotion (Figure 4).

Receptor dimerization between the GHS-R1a and other receptors include the formation of dimers between the GHS-R1a and serotonin $5HT_{2c}$ receptors, melanocortin 3 and 4 (MC3, MC4) receptors, somatostatin receptors and oxytocin receptors [90]. In the case of $5HT_{2c}$/GHS-R1a dimers, activation of $5HT_{2c}$ results in the inhibition of ghrelin induced $G\alpha_{q/11}$ signaling, whereas blocking the $5HT_{2c}$ enhances GHS-R1a signaling and ghrelin induced signaling [91]. The formation of oxytocin receptor (OTr)/GHS-R1a dimers is linked to decreased oxytocin-mediated $G\alpha_{q/11}$ signaling [92]. In all, the promiscuity of the GHS-R1a

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and the diverse signaling modalities that these promiscuous interactions produce require further investigation.

The interaction between the GHS-R1a and other membrane proteins is not restricted to G-coupled protein receptors. Recent evidence shows that the GHS-R1a forms complexes with the melanocortin accessory protein 2 (MRAP-2), a single transmembrane protein highly expressed in the hypothalamus and important for the regulation of MC4 signaling [93]. *In Vitro* assays show that the GHS-R1a and MRAP-2 form complexes that enhance ghrelin stimulated $G\alpha_{q/11}$ signaling as measured by IP3 production [94]. *In Vivo*, MRAP-2 KO mice show attenuated feeding responses and Fos expression and do not show increased pAMPK expression in the ARC following ghrelin treatment [94]. These data suggest that GHS-R1a/MRAP-2 complexes in the ARC and specifically in AGRP neurons, are critical for the orexigenic effects of ghrelin [94]. Whether GHS-R1a/MRAP-2 complexes occur in other brain regions remains to be determined.

Recent data suggest that the liver-expressed antimicrobial peptide 2 (LEAP2) is a negative feedback regulatory protein that acts on the GHS-R1a as an endogenous ligand with inverse agonist/antagonist properties [95–98]. In contrast to ghrelin, circulating levels of LEAP2 decrease following a fast and increase postprandially. Moreover, LEAP2 attenuates the activation of GHS-R1a in cell culture and reduces ghrelin induced food intake and growth hormone secretion in animal studies. Not surprisingly, blocking LEAP2 enhances ghrelin induced growth hormone secretion [95]. A recent paper demonstrates that the ratio of LEAP2/acyl ghrelin is an accurate indicator of GHSR signalling with a greater levels of LEAP2 over ghrelin leading to ghrelin resistance [96]. These data support LEAP2 acting as a negative feedback signal to the GHS-R1a and could be a potential target for drugs regulating energy balance. Nevertheless, it is not known if LEAP2 can cross the blood brain barrier and affect GHS-R1a in the CNS. These are potentially intriguing possibilities as LEAP2 could not only be used to treat metabolic disorders but also as a treatment for addiction, just as some synthetic GHS-R1a antagonists, inverse agonists, and ghrelin vaccines that peripherally sequester ghrelin are currently being considered [99, 100].

Concluding Remarks and Future Perspectives

Ghrelin plays a unique role in energy metabolism while influencing a wide range of systems within the body. Ghrelin signaling through the GHS-R1a receptor requires ghrelin acylation with a medium-chain fatty acid, placing this rare serine posttranslational modification as a potential control point for modulating ghrelin-dependent physiological processes (see Outstanding Questions). The unravelling of the complex mechanisms through which ghrelin and the GHS-R1a receptor act in the brain to modulate behavior clearly implicate ghrelin as an important target for treating a number of conditions including obesity, metabolic disorders, stress and anxiety disorders, and addiction. Since its discovery, GHS-R1a was identified as a primary therapeutic target. However, given the diversity of GHS-R1a signaling mechanisms and its promiscuous interaction with other G-coupled protein receptors and membrane proteins, it is difficult to envision drugs that will selectively reduce appetite through GHS-R1a antagonism without causing undesired side effects. Indeed, while GHS-R1a antagonists can reduce weight and food intake, they also increase vulnerability to

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develop anxiety and depressive like symptoms following stress, an effect reported in clinical trials for other drugs like the CB-1 receptor antagonist rimonabant.

In contrast, GOAT appears to be an excellent therapeutic target given that this enzyme does not appear to modify any protein targets beyond ghrelin. While GOAT and ghrelin KO mice have demonstrated that negative metabolic and neurological consequences can result from a complete loss of acyl ghrelin in circulation [101], the ability to reduce and control acyl ghrelin concentrations with selective GOAT inhibitors in a dosage- and time-dependent manner could present an avenue for the treatment of obesity, Type II diabetes and even addiction. Indeed, the ability to target GOAT without crossing the blood-brain barrier provides an additional pharmacological benefit not enjoyed by molecules acting on the GHS-R1a receptor.

In all, the data reviewed illustrate to the complex biology of the ghrelin system and provide for insight into potential avenues that could lead to the design of compounds that modulate the secretion of active ghrelin or modulate GHS-R1a receptor signaling for the treatment of metabolic disorders. The continuing application of multidisciplinary research spanning medicinal chemistry, biochemistry, cellular signal transduction, and neuroendocrinology is essential to both understand and exploit ghrelin signaling for therapeutic advantage.

Acknowledgements

Funding was provided by a grant from the Canadian Institutes for Health Research awarded to AA and grants from the American Diabetes Association (#1-16-JDF-042), the National Institutes of Health (GM134102), and Syracuse University to JLH.

Glossary

2-Arachidonoylglycerol
2-AG. Endogenous cannabinoid that binds the CB1 receptor to influence appetite and other processes

Agouti related peptide
AGRP. Peptide produced by cells in the ARC that also produce NPY, and one with a potent orexigenic effect

Adenosine monophosphate-activated protein kinase
(AMPK). Important kinase in the regulation of intracellular energy production

Area Postrema
AP. Brain stem region that lies outside of the blood brain barrier and important for nutrient sensing

Diacylglycerol
DAG. Intracellular molecule stimulated by Gq G-coupled protein signaling and one that can serve as a substrate for the endogenous cannabinoid 2-AG

Diacylglycerol lipase
DAGL. Enzyme that converts DAG into the endogenous cannabinoid 2-AG

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Dopamine receptor 1
DRD1. G-coupled protein receptor associated with the effects of dopamine on cognition and reward

Dopamine receptor 2
DRD2. G-coupled protein receptor associated with the effects of dopamine on feeding and metabolism

Ghrelin
Ghrl. Peptide ligand for GHS-R1a, linked to metabolic regulation

GO-CoA-Tat
Peptide-based inhibitor of GOAT, composed of regions mimicking both ghrelin and octanoyl-CoA with an attached Tat peptide sequence to enable cell penetration

Growth Hormone Secretagogue Receptor 1a
GHS-R1a. Only known ghrelin receptor

Ghrelin-O-acyltransferase
GOAT. Enzyme required for the production of the active form of ghrelin, catalyzes a unique serine octanoylation posttranslational modification

Hypothalamic Arcuate Nucleus
ARC. Hypothalamic region important for the regulation of feeding and energy balance and rich in GHS-R1a

Liver expressed anti-microbial peptide 2
LEAP2. Endogenous GHS-R1a antagonist

Melanocortin accessory protein 2
MRAP-2. Membrane protein important for melanocortin receptor signaling, and recently associated with GHS-R1a signaling

Neuropeptide Y
NPY. Peptide produced by the gut and the brain. NPY is produced in the ARC and from here, it targets a number of brain regions to stimulate appetite and decrease energy expenditure

Nucleus of the Solitary Tract
NTS. Brain stem region that integrates hormonal information with signals ascending form vagal afferents. This region contains GHS-R1a

Phospholipase C
PLC. Enzyme associated with G-coupled protein signaling cascades

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Highlights

- Ghrelin octanoylation by ghrelin *O*-acyltransferase represents a key control point in metabolic signaling
- Recent advances in GOAT inhibitor development offer the potential to target ghrelin signaling through GOAT for therapeutic effect
- Discovery of LEAP2, an endogenous GHS-R1a antagonist secreted by the liver.
- Identification of proteins that interact with GHS-R1a to modulate receptor activation expands the landscape of ghrelin-GHS-R1a signaling.
- Discovery of the ability of the GHS-R1a to dimerize and alter signaling of other G-coupled protein receptors.

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Outstanding Questions Box

- How does GOAT bind, recognize, and octanoylate ghrelin? Defining the structure of GOAT and other MBOAT family acyltransferases is an essential step for understanding these enzymes, the secreted proteins they modify, and the roles they play in autocrine, paracrine, and endocrine signaling.
- What is the most efficient route for developing GOAT inhibitors for modulating ghrelin-dependent signaling pathways? As the field begins to build beyond product- and substrate-mimetic inhibitors, what will be the best strategy for small molecule inhibitor development? As we define the structure and mechanism of GOAT, can we exploit mechanism-based inhibition against this enzyme?
- How does GHS-R1a respond to ghrelin and other molecules? Does the GHS-R1a require ghrelin for signaling? Are there other endogenous ligands that alter GHS-R1a signaling?
- Does GHS-R1a interact with other receptors or membrane proteins to bias intracellular signaling? Are interactions important for *In Vivo* physiological mechanisms? Can understanding these interactions be useful in future treatment for obesity?

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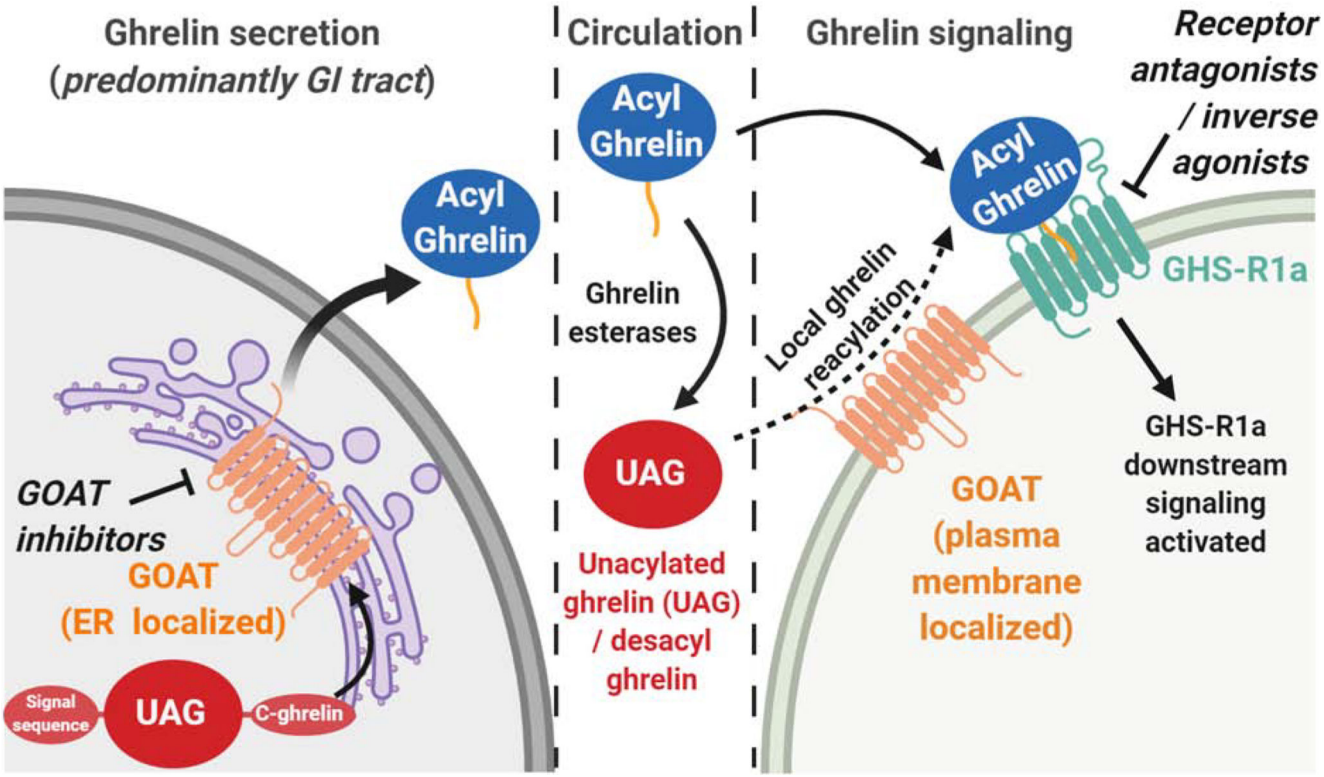

Figure 1. Ghrelin is activated for biological signaling by ghrelin *O*-acyltransferase (GOAT).

Following expression in endocrine cells, ghrelin undergoes processing including serine octanoylation by GOAT (*orange tail*) prior to secretion into the bloodstream. In circulation, esterases can remove the octanoyl group to produce unacylated ghrelin (UAG). When reaching a cell expressing the GHS-R1a receptor, acylated ghrelin can bind and activate downstream signaling. In some cell types, plasma membrane-exposed GOAT has been proposed to catalyze re-acylation of UAG to generate ghrelin which can then activate GHS-R1a signaling.

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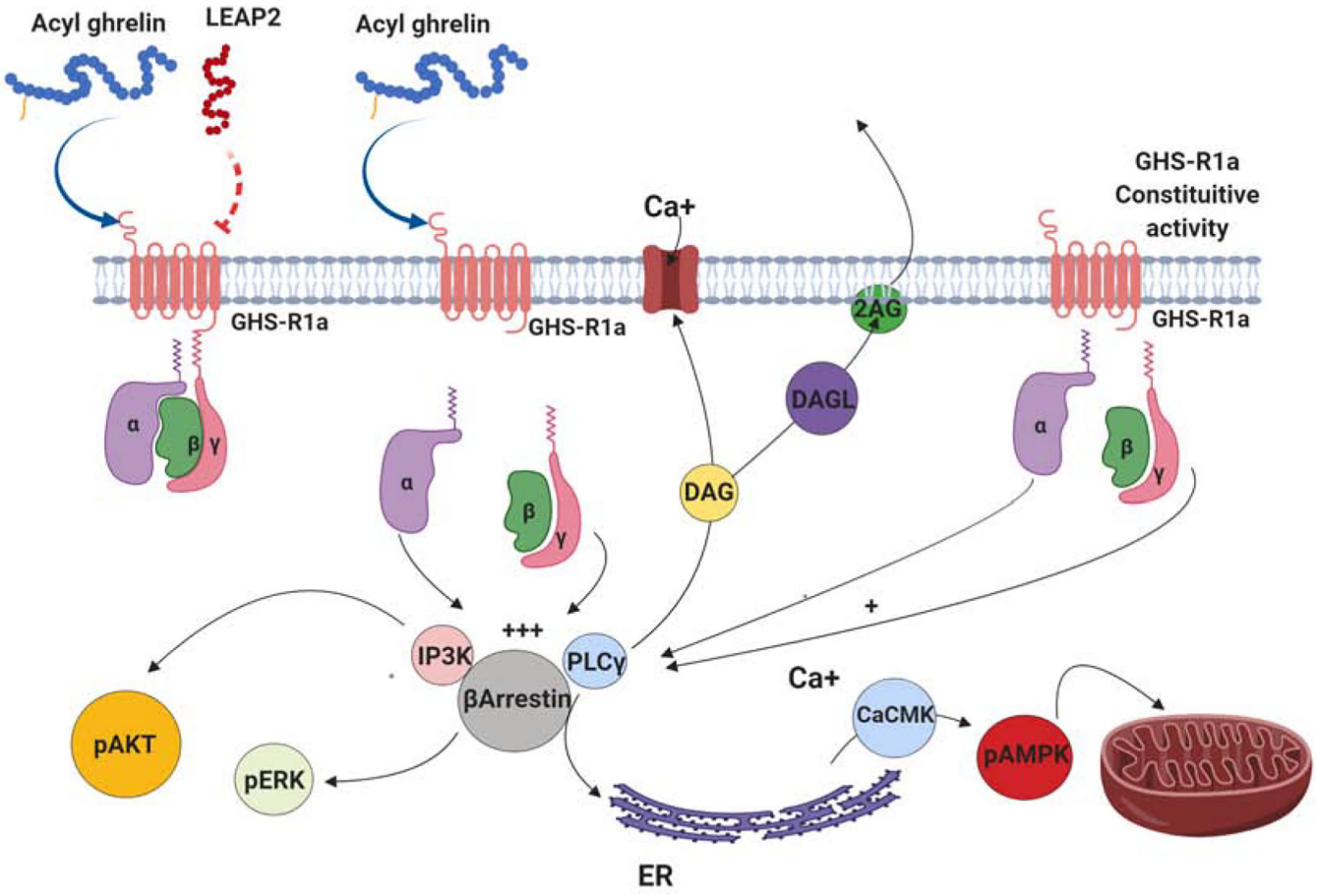

Figure 2. GHS-R1a signaling.

Ghrelin and the liver-expressed antimicrobial peptide 2 (LEAP2) are endogenous ligands for GHS-R1a, with ghrelin acting as an agonist and LEAP2 acting as an antagonist. GHS-R1a is an αq G-coupled protein receptor that, upon activation, induces the activation of several intracellular signaling pathways including the activation and accumulation of phospholipase C γ (PLCγ), the activation of β-arrestin, and the accumulation of inositol phosphatase 3 kinase (IP3K). Activation of GHS-R1a also results in the production of diacylglycerol (DAG), an intracellular signal that in the presence of diacylglycerol lipase (DAGL) is converted into the endocannabinoid 2-arachidonoylglycerol (2-AG). Increased concentrations of PLCγ stimulate the release of Ca^{+} from the endoplasmic reticulum, with higher Ca^{+} concentrations then facilitating calcium/calmodulin kinase (CaCMK)-catalyzed phosphorylation of adenosine monophosphate-activated protein kinase (pAMPK). The presence of pAMPK is associated with many of the metabolic effects of ghrelin induced GHS-R1a activation. In the absence of ghrelin, GHS-R1a has relatively high constitutive activity that can be reduced by treatment with inverse agonists. Finally, GHS-R1a stimulation is also associated with increased phosphorylation of ERK via the β-arrestin pathway, and increase AKT phosphorylation via increased levels of PI3K.

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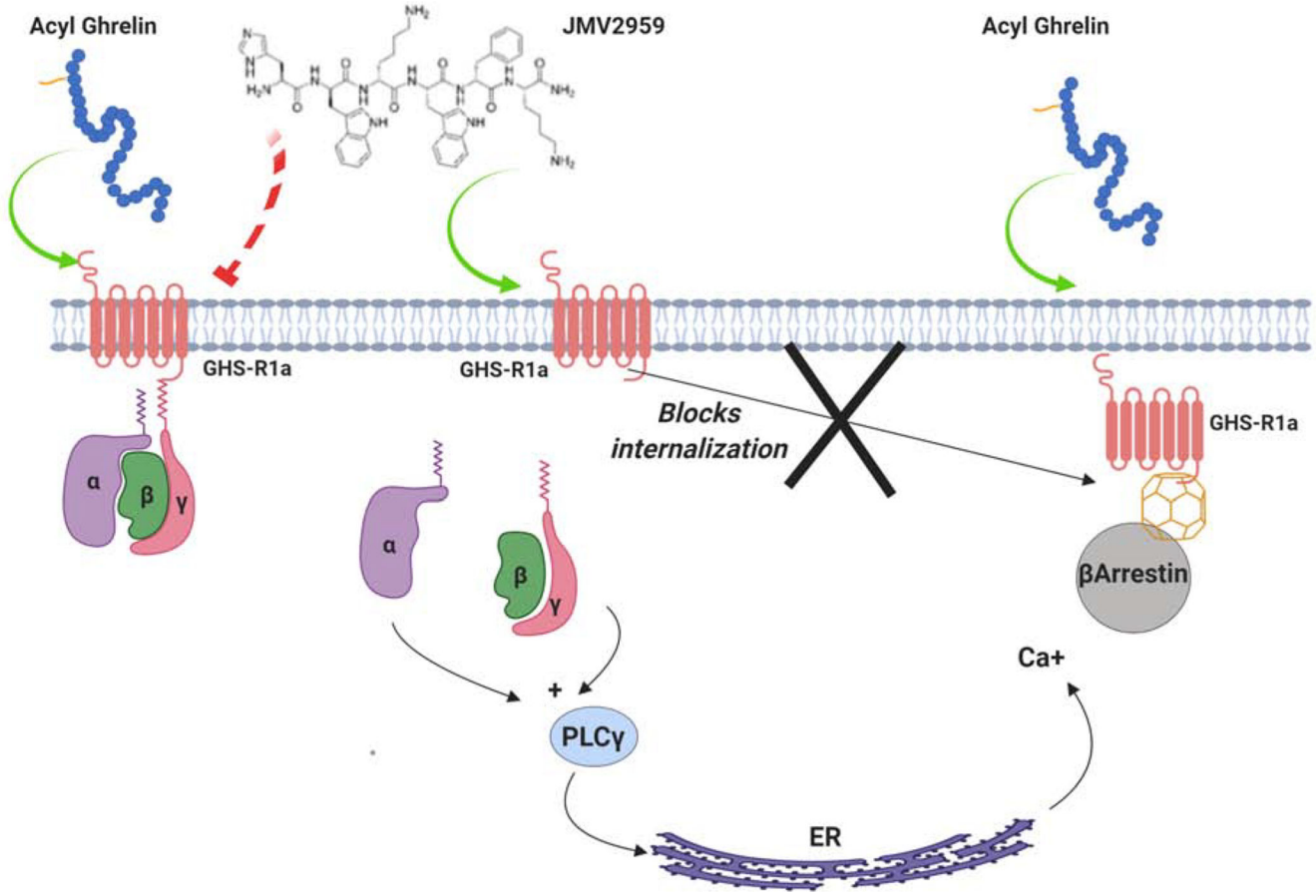

Figure 3. GHS-R1a agonists and antagonists.

The activation of GHS-R1a can be blocked by a number of compounds that act as antagonists in the presence of ghrelin. One such molecule is JMV2959, a commonly used compound that blocks ghrelin induced GHS-R1a signaling. This compound, however, acts as a partial agonist in the absence of ghrelin and can activate Gαq signaling through GHS-R1a. At the same time, JMV2959-induced activation prevents GHS-R1a internalization provoked by the activation of the β-arrestin pathway, potentially preventing GHS-R1a desensitization.

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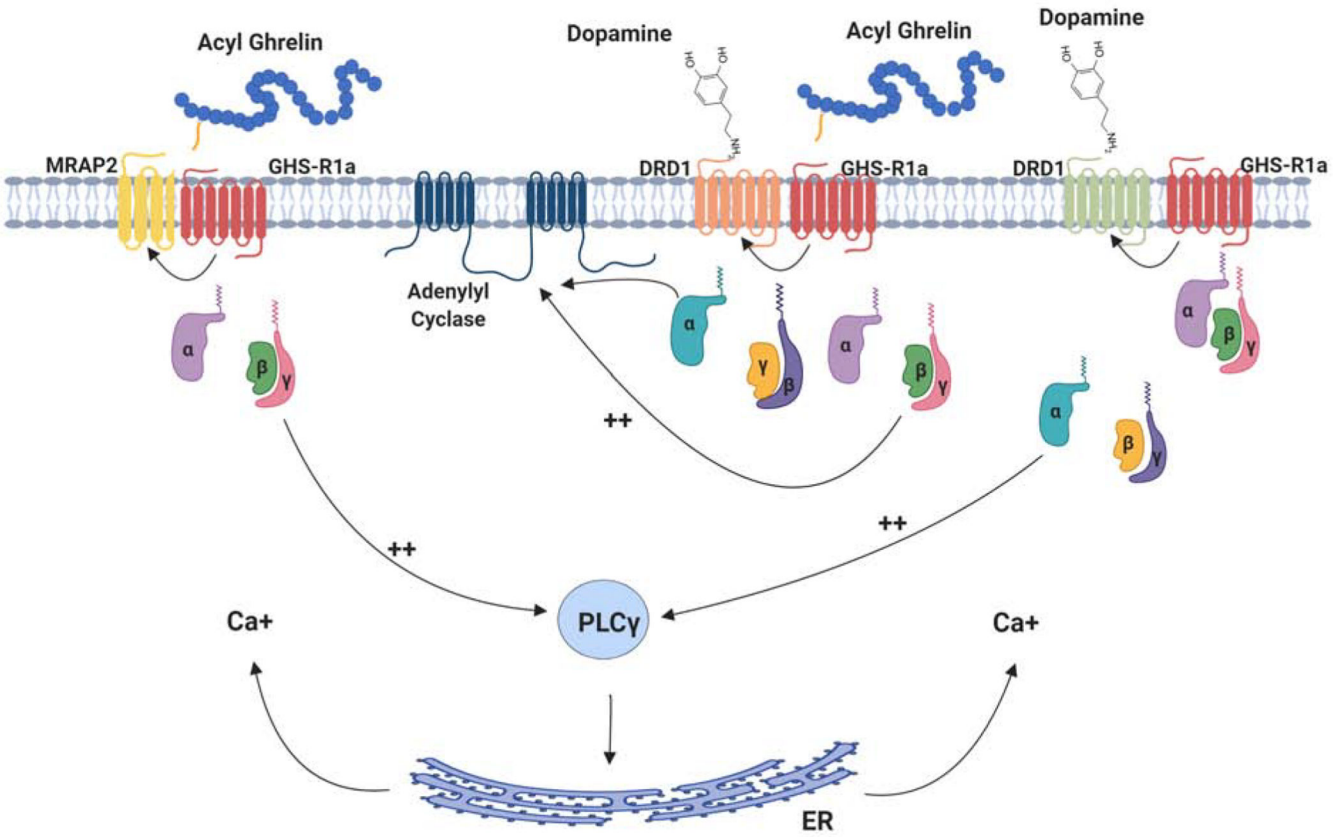

Figure 4. GHS-R1a dimerizes with other GPCRs to bias receptor signaling.

GHS-R1a can form dimers with a number of other integral membrane proteins in the presence or absence of ghrelin. For example, GHS-R1a can dimerize with the melanocortin accessory protein 2 (MRAP-2), a protein that facilitates melanocortin receptor signaling. GHS-R1a also dimerizes with dopamine D1 and D2 receptors and can bias their downstream signaling in both ligand-dependent and ligand-independent manners.