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THE UPS AND DOWNS OF GROWTH HORMONE SECRETAGOGUE RECEPTOR SIGNALING

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Running Title: Regulation of GHSR signaling by ghrelin and LEAP2

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Abbreviations

AMP – adenosine monophosphate

AMPK – 5' AMP-activated protein kinase

BMI – body mass index

DAG – diacylglycerol

EC_{50} – half maximal effective concentration

ERK1/2 – extracellular signal-regulated kinases 1/2

GDP – guanosine diphosphate

GH – growth hormone

GHSR – growth hormone secretagogue receptor

GOAT – ghrelin-*O*-acyl transferase

GPCR – G protein-coupled receptor

GTP – guanosine triphosphate

IP1 – inositol monophosphate

IP3 – inositol 1,4,5-trisphosphate

LEAP2 – liver-expressed antimicrobial peptide 2

MRAP-2 – melanocortin receptor accessory protein 2

mRNA – messenger RNA

NMR – nuclear magnetic resonance

PC1/3 – prohormone convertase 1/3

PLC – phospholipase C

VOCCs – voltage-operated calcium channels

Keywords: GHSR, ghrelin, LEAP2, food intake regulation, glucose homeostasis.

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ABSTRACT

The growth hormone secretagogue receptor (GHSR) has emerged as one of the most fascinating molecules from the perspective of neuroendocrine control. GHSR is mainly expressed in the pituitary and the brain, and plays key roles regulating not only growth hormone secretion but also food intake, adiposity, body weight, glucose homeostasis as well as other complex functions. Quite atypically, GHSR signaling displays a basal constitutive activity that can be up- or down-regulated by two digestive system-derived hormones: the octanoylated-peptide ghrelin and the liver-expressed antimicrobial peptide 2 (LEAP2), which was recently recognized as an endogenous GHSR ligand. The existence of two ligands with contrary actions indicates that GHSR activity can be tightly regulated and that the receptor displays the capability to integrate such opposing inputs in order to provide a balanced intracellular signal. This article provides a summary of the current understanding of the biology of ghrelin, LEAP2 and GHSR, and discusses the re-conceptualization of the cellular and physiological implications of the ligand-regulated GHSR signaling, based on the latest findings.

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1. INTRODUCTION

The growth hormone secretagogue receptor (GHSR) was initially described as a target of synthetic compounds with growth hormone (GH)-releasing properties [1]. The observation that GHSR is found not only in the pituitary and the hypothalamus but also in a wide variety of brain areas and peripheral tissues early suggested that this receptor played additional physiological roles beyond its canonical role regulating GH secretion [1,2]. The subsequent discovery of the stomach-derived peptide ghrelin as an endogenous agonist of GHSR [3], and the study of its functions, clearly showed that GHSR also modulates a plethora of physiologic processes including food intake, adiposity, body weight, glucose homeostasis, autonomic nervous system activity as well as complex cognitive functions such as reward-related behaviors [4]. Recently, the liver-expressed antimicrobial peptide 2 (LEAP2) was recognized as another ligand of GHSR that abrogates its activity [5]. Thus, GHSR signaling depends on two circulating ligands, which play contrary actions on the receptor activity and tightly modulate its physiological impact. Given the capability of GHSR to regulate fundamental physiological functions, this receptor is considered an attractive therapeutic target to treat a variety of human disorders including cachexia and alcohol-use disorder [6,7]. However, the pharmaceutical development of safe GHSR-based therapies requires a precise understanding of the intricate mechanisms that apparently control its activity. This review summarizes the current knowledge of the biology of ghrelin, LEAP2 and GHSR in mammals, and discusses the implications of the ligand-regulated GHSR signaling at cellular and physiological levels.

2. GHRELIN

2.1 Biosynthesis

The gene encoding ghrelin contains five exons and four introns in rodents and humans [8]. The gene encoding ghrelin is highly expressed in endocrine cells (hereafter named ghrelin cells) of the stomach oxyntic mucosa, moderately expressed in duodenum, jejunum, ileum and colon, and not expressed in the brain [3,9]. The biosynthetic pathway for ghrelin is well defined (**Figure 1**). The mRNA encoding ghrelin is translated into a 117-residue precursor, named preproghrelin. In the endoplasmic reticulum, preproghrelin is first cleaved by a signal peptidase into a 24-residue N-terminal signal peptide and preproghrelin$_{25-117}$ (or proghrelin) [10]. Proghrelin is then acylated at Ser^{3} by ghrelin-*O*-acyl transferase (GOAT), a transmembrane enzyme mainly located in the endoplasmic reticulum [11,12]. Lipid substrates of GOAT include short- to mid-chain fatty acids

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(ranging from acetate to tetradecanoic acid) thio-esterified with Coenzyme A [11]. *In vivo*, proghrelin octanoylation is the most frequent detected modification, presumably due to the type of ingested lipids [13]. Octanoylated-proghrelin is then cleaved by prohormone convertase 1/3 (PC1/3) in the Golgi apparatus to obtain the acylated N-terminal 28-residue preproghrelin$_{25\text{-}52}$, which corresponds to mature ghrelin, and the C-terminal preproghrelin$_{53\text{-}117}$ [10]. Preproghrelin$_{53\text{-}117}$ is further cleaved, presumably by PC1/3, to generate preproghrelin$_{76\text{-}98}$, a 23-residue peptide named obestatin [14]. The biological significance of obestatin and the existence of a specific receptor for this peptide are yet a topic of debate [15]. Ghrelin's amino acid sequence is highly conserved across species, with its ten N-terminal residues being identical in all mammals [8]. The N-terminal residues of ghrelin, including the octanoyl moiety, are required and sufficient to induce GHSR activation *in vitro* [16,17] highlighting its role for ghrelin's bioactivity. The C-terminal residues of ghrelin, in contrast, do not interact with GHSR but seem to be required for the full hormone's bioactivity *in vivo*, presumably because they protect the peptide from degradation in plasma [18].

2.2 Plasma level regulation

Across the biosynthetic process in the regulated secretory pathway, proghrelin-derived peptides are sorted from the Golgi apparatus into secretory granules [19]. Upon stimulation, secretory granules fuse with the plasma membrane in a calcium-dependent manner and proghrelin-derived peptides are secreted [19]. *In vitro*, ghrelin is mainly secreted upon norepinephrine-induced activation of β1-adrenergic receptors, which are highly expressed in ghrelin cells [20]. Ghrelin secretion *in vitro* also increases in response to gastric inhibitory peptide and glucagon, while it decreases in response to glucose, leptin, insulin, glucagon-like peptide 1, gastrin and somatostatin [21–23]. *In vivo*, plasma ghrelin levels vary according to the feeding schedule, rising before meals and decreasing to baseline after food intake [24]. The post-prandial decrease of ghrelin depends on the caloric content and the type of nutrients ingested, with carbohydrates being stronger inhibitors than proteins and lipids [24]. In rats, glucose infusion potently decreases plasma ghrelin levels [25], while infusion of lipids and amino acids moderately decrease ghrelin levels [26]. Ghrelin levels increase under energy deficit conditions such as fasting, calorie restriction, anorexia nervosa or cachexia [27,28]. The observation that the fasting-induced increase in ghrelin levels is abrogated in mice treated with either the β1-adrenergic receptor antagonist atenolol or the blocker of catecholaminergic neurotransmission reserpine indicates that the increase in ghrelin levels under energy deficit conditions depends on the activation of the sympathetic nervous system [20,29]. In line with this notion, caloric restriction-

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induced elevation of ghrelin levels is impaired in mice lacking β1-adrenergic receptors in ghrelin cells [29]. Ghrelin levels also rise in humans and rodents under several types of stress [30–32]. Atenolol treatment of chronic social defeat-stressed mice lowers plasma ghrelin levels, suggesting that the stress-induced elevation of ghrelin also depends on β1-adrenergic receptor signaling [33].

Plasma ghrelin is bound to lipoproteins and albumin [34,35], and its half-life is estimated in ~9-13 minutes in humans [36]. Plasma ghrelin can be either degraded by proteases or des-acylated by serum esterases [37,38]. Ghrelin des-acylation generates desacyl-ghrelin, whose plasma levels are ~5-to-20-fold higher than ghrelin [39,40]. Some evidence indicates that desacyl-ghrelin is also produced in the stomach and that its secretion is regulated by norepinephrine and glucose *in vitro*, in a similar extent to what was shown for ghrelin [41]. In addition, some studies showed that desacyl-ghrelin acts in the brain and regulates feeding behavior, adipogenesis, glucose metabolism and thermoregulation [41–44]. However, the biological role of desacyl-ghrelin is a matter of controversy because it does not bind to GHSR at physiological relevant concentrations [17] and its specific receptor is not yet reported. Some recent evidence suggests that circulating desacyl-ghrelin could be extracellularly acylated, in order to generate ghrelin, by GOAT located in the plasma membrane of bone marrow and hippocampal cells [45,46], but this possibility requires further confirmation. In summary, many aspects of the mechanisms controlling plasma ghrelin concentration still remain to be clearly elucidated.

3. LEAP2

3.1 Biosynthesis

The gene encoding LEAP2 in humans and rodents consists of three exons and two introns [47]. The biosynthetic pathway for LEAP2 remains to be fully characterized, but different studies allow to propose a model (**Figure 1**). PCR analysis in mouse and rat tissues showed that LEAP2 mRNA is mainly found in liver and jejunum, but also in the duodenum and ileum [5,48]. In humans, several spliced variants of mRNAs encoding LEAP2 precursors have been described, including a fully spliced mRNA that encodes mature LEAP2 in the liver and gastrointestinal tract, and intron-containing variants found in different gastrointestinal tissues [49]. In humans, the fully spliced mRNA encoding LEAP2 is translated into a 77-residue precursor, preproLEAP2, which is first cleaved by a signal peptidase into a 22-residue N-terminal signal peptide and

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preproLEAP2$_{23-77}$ (or proLEAP2) [47]. Then, proLEAP2 is processed into a preproLEAP2$_{23-37}$ and preproLEAP2$_{38-77}$, which corresponds to the 40-residue mature LEAP2 and is the main variant of LEAP2 found in mouse liver and small intestine [5]. The proposed proLEAP2 processing enzyme is furin, a calcium-dependent serine endoprotease that cleaves peptides at the C-terminal end of paired basic residues and usually processes precursors in the constitutive secretory pathway [50]. A recent study found that LEAP2 is also processed into LEAP2$_{38-47}$ in the intestinal epithelium of humans and mice [51]. LEAP2's amino acid sequence is highly conserved in mammals, particularly the N-terminal end, where the initial six residues are identical across species [52,53]. The N-terminal region of LEAP2 includes several hydrophobic residues and is essential for GHSR binding [54–56]. The C-terminal end of LEAP2, which mainly clusters positively charged residues and includes two pairs of disulfide bonds: Cys^{54}-Cys^{65} and Cys^{60}-Cys^{70}, does not interact with GHSR and likely contributes to the peptide stability in plasma [47,52].

The cell types producing LEAP2 and the mechanisms controlling its biosynthesis and secretion are just beginning to be explored and some apparent discrepancies seem to exist. *In situ* hybridization studies in mice showed that LEAP2 mRNA is moderately expressed in hepatocytes and highly expressed in jejunum enterocytes along the luminal surface of the villi, without expression in the lamina propria or crypts [5]. Other studies in mice found that LEAP2 mRNA is expressed in enteroendocrine and epithelial cells of the jejunum while is absent in the colon [51,57]. In humans, LEAP2 mRNA was found in enteroendocrine cells and in epithelial cells lining the colon crypts, and LEAP2 immunoreactivity was detected in the colon crypts with a whole-cell staining pattern suggestive of cytoplasmic localization [49,51,58]. *In vitro* studies showed that LEAP2 gene expression can be regulated in hepatocytes. LEAP2 mRNA levels were found increased or decreased in response to high cholesterol sera and poly-unsaturated fatty acids, respectively, in a human hepatoma cell line [59]. Also, ghrelin was shown to reduce LEAP2 mRNA levels in primary cultures of mouse hepatocytes and in a murine hepatoma cell line via a 5' AMP-activated protein kinase (AMPK)-dependent pathway [48]. To our knowledge, the mechanisms controlling LEAP2 secretion have not been yet elucidated, but it is likely that LEAP2 secretion is regulated in a tissue-specific manner since hepatocytes mainly secrete peptides via the constitutive secretory pathway [60], while enterocytes and enteroendocrine cells usually secrete peptides via the regulated secretory pathway [61].

3.2 Plasma level regulation

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Few studies investigated the biology of LEAP2 *in vivo*. Originally, LEAP2 was shown to exhibit antimicrobial activity *in vitro*, which gave its name [47]. However, plasma LEAP2 levels range concentrations that could affect GHSR activity, as discussed below, but are several orders of magnitude below the levels required for its antimicrobial effects [5]. Thus, it is more likely that physiological levels of LEAP2 play a major role regulating GHSR activity *in vivo*. In this regard, plasma LEAP2 levels fluctuate in an inverse pattern compared to ghrelin levels. In particular, plasma LEAP2 levels decrease upon fasting and return to baseline after refeeding in both mice and rats [5,48,62]. LEAP2 mRNA levels were found decreased in the liver, but not in the jejunum or the ileum, of fasted rats [48], while LEAP2 mRNA remained unchanged in the liver of fasted mice [62]. Fasting-induced decrease of LEAP2 levels may depend on glucose and ghrelin levels since administration of glucose to fasted mice increases plasma LEAP2 levels [62], while ghrelin treatment decreases plasma LEAP2 and liver LEAP2 mRNA levels [48]. In contrast to ghrelin, plasma LEAP2 levels also decrease upon calorie restriction [5]. Interestingly, overexpression of LEAP2 impairs calorie restriction-induced increase of ghrelin levels in mice, suggesting that LEAP2 inhibits the biosynthesis or secretion of ghrelin [5]. LEAP2 mRNA levels were found low in the duodenum of mice subjected to bariatric surgery [5], but unchanged in the liver or jejunum of diet-induced obese mice, which show increased plasma LEAP2 levels, as discussed below [62]. Interestingly, systemically-injected LEAP2 is rapidly degraded in plasma, with a half-life estimated in ~15 minutes [5]. $LEAP2_{38-47}$ was also detected in human plasma samples, but its levels are in the picomolar range far below the levels of intact LEAP2 [51]. Thus, the cell type and the mechanisms regulating the fluctuations of LEAP2 levels in plasma are just beginning to be clarified.

4. GHSR

4.1 Biology and structure

Ghrelin and LEAP2 act via GHSR, which is a G protein-coupled receptor (GPCR) that possesses seven membrane-spanning α-helical domains with its N- and C-terminal ends located extra- and intracellularly, respectively [63]. The gene encoding GHSR consists of two exons and one intron [64]. Exon 1 codes the 5′ untranslated region, the extracellular domain and transmembrane regions I-V, while exon 2 codes for transmembrane regions VI and VII, and the intracellular domain. The fully spliced GHSR mRNA encodes the 366-residue receptor containing all seven transmembrane domains that possesses high binding affinity for ghrelin. The non-spliced GHSR mRNA generates a truncated 289-residue version of GHSR, encoded by exon 1

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and part of the intronic region, that does not bind ghrelin but was found to bind and retain full-length GHSR in the endoplasmic reticulum in heterologous expression systems [64,65].

The recently solved crystal structure of GHSR bound to a synthetic antagonist unmasked the putative ligand binding sites of this receptor [66]. In particular, two cavities are observed in GHSR structure that could act as binding pockets for ghrelin (**Figure 2A**). In addition, GHSR has a gap (or crevasse) formed by a cluster of hydrophobic residues, particularly five Phe residues of the transmembrane domains VI and VII, which presumably recognize the octanoyl moiety of ghrelin [66,67]. GHSR seems to mainly interact with the N-terminal region of ghrelin, which contains a hydrophobic core that includes the octanoyl moiety and the residues Phe^4 and Leu^5 [67,68] (**Figure 2B**). GHSR also interacts with the N-terminal region of LEAP2, which is sufficient to reduce GHSR activation and contains the hydrophobic residues Met^1, Pro^3, Phe^4 and Trp^5 [56,69,70] (**Figure 2C**). The presence of two disulfide bonds in LEAP2 is also relevant for its binding to GHSR [56,69,70]. GHSR displays high and equivalent affinity for ghrelin and LEAP2, with affinity constants in the order of ~1 nM [56,70]. Interestingly, GHSR activity is not only controlled by ghrelin and LEAP2 but also regulated via ligand-independent mechanisms, as discussed below (**Figure 3**).

4.2 Ghrelin-evoked GHSR activity

GHSR is recognized as a promiscuous receptor that controls the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in different heterotrimeric G protein complexes and results in the activation of Gα-GTP and Gβγ subunits [71,72]. Specifically, GHSR can couple to G protein complexes containing either $G\alpha_q$, $G\alpha_{i/o}$, $G\alpha_{12/13}$ or $G\alpha_s$ subunits, hereafter named G_q, $G_{i/o}$, $G_{12/13}$ or G_s proteins, respectively [71,72]. Classically, ghrelin-evoked GHSR activity was shown to recruit G_q protein and modulate the activity of the membrane-associated phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate in order to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which, in turn, releases calcium from the endoplasmic reticulum to the cytoplasm. DAG and calcium activate protein kinase C β that, among other effects, inhibits potassium channels and depolarizes excitable cells. For instance, ghrelin-evoked G_q protein signaling induces depolarization of neurons via inhibition of voltage-gated potassium channels type 7 [73]. GHSR-mediated increments of intracellular calcium also up-regulate other calcium-sensitive pathways, such as calcium/calmodulin-dependent protein kinase II that phosphorylates and activates AMPK which, in turn, modulates gene transcription and neurotransmission [74]. In parallel, ghrelin-evoked G_q protein signaling

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inhibits presynaptic voltage-operated calcium channels (VOCCs), reducing neurotransmission, and postsynaptic VOCCs that would reduce neuronal excitability [75,76]. GHSR also recruits $G_{i/o}$ protein signaling that, in turn, activates the phosphoinositide 3-kinase cascade and leads to extracellular signal-related kinases 1 and 2 (ERK1/2) activation [72]. Alternatively, ghrelin-evoked GHSR activity activates ERK1/2 via G_q protein- and β-arrestin-dependent mechanisms [74,77]. Ghrelin-evoked GHSR activity was also shown to modulate G_s protein signaling, which increases cyclic AMP production and activates the protein kinase A pathway [78,79], and to induce RhoA kinase activity via $G_{12/13}$ protein coupling [80]. Finally, ghrelin binding to GHSR induces internalization of the ligand-receptor complex in a β-arrestin-dependent manner [81].

4.3 Ligand-independent GHSR actions

Current understanding of GHSR signaling considers that this receptor explores multiple conformational states that operate in thermodynamic equilibrium, as is the case of most GPCRs [82]. Such equilibrium of GHSR conformational states is dynamic and strongly affected by GHSR ligands, which bind to a subset of conformational states and elicit a population shift that establishes a new equilibrium. Thus, binding of ghrelin to GHSR shifts the equilibrium towards an active conformational state of GHSR that, in turn, triggers a variety of intracellular events, as described above [83]. In contrast, LEAP2 drives the equilibrium towards an inactive state [56]. Importantly, GHSR adopts two major conformational states in the absence of ligands: an inactive state, in which the receptor is associated to a GDP-loaded G_q protein in an inactive preassembled complex, and an active state, where the receptor triggers GDP-to-GTP exchange [83]. Such active conformational state is responsible for the constitutive GHSR activity that induces intracellular signaling in a ligand-independent manner [83]. In *in vitro* settings and in the absence of ligands, constitutive GHSR activity induces G_q protein signaling that results in intracellular accumulation of the IP_3 metabolite IP_1 equivalent to ~50% of its maximal capacity [63]. Constitutive GHSR signaling was also shown to recruit G_s and $G_{i/o}$ proteins signaling [63,75,84]. Via $G_{i/o}$ protein, constitutive GHSR activity inhibits VOCCs trafficking to the plasma membrane, in a Gβγ subunit- and voltage-independent manner, and reduces neurotransmission at the presynaptic level [75,84,85]. In contrast to ghrelin-evoked GHSR signaling, constitutive GHSR signaling triggers the internalization of the receptor in a β-arrestin-independent manner [81,86]. The ability of GHSR to adopt conformational states that induce constitutive signaling makes it susceptible not only to the action of antagonist but also to the action of inverse agonist ligands, which suppress its constitutive activity.

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GHSR also displays the capability to interact with other proteins. Specifically, GHSR has been shown to form heterodimers with other GPCRs, including the receptors for either dopamine, serotonin, oxytocin, orexin or somatostatin [87–89]. The formation of oligomers between GHSR and other GPCRs not only allows a cross-talk between the signaling pathways of each receptor but also enables a mutual allosteric regulation between both GPCRs that could affect specific features, including the signaling cascades recruited or the binding properties to their ligands [90]. For instance, coupling to the serotonin receptor subtype 2C itself attenuates ghrelin-evoked GHSR activation [91], while GHSR itself biases the ligand-evoked activation of the dopamine receptor 2 to a non-canonical G_q protein signaling [83,92]. Moreover, GHSR interacts with the melanocortin receptor accessory protein 2 (MRAP-2), a 205 residues single-pass transmembrane protein that enhances ghrelin-evoked GHSR activation via G_q protein signaling but reduces constitutive GHSR activity [93,94].

4.4 LEAP2 action on GHSR

LEAP2 impairs both ghrelin-evoked and constitutive GHSR activities. Initially, LEAP2 was shown to dose-dependently decrease the maximal effect of ghrelin on GHSR-mediated activation of G_q protein and β-arrestin pathways, without any effect on the EC_{50}, suggesting that LEAP2 acts as a non-competitive antagonist that allosterically regulates GHSR [5]. However, two subsequent independent studies using binding competition, measurement of IP_1 and calcium mobilization assays found that LEAP2 dose-dependently increased the EC_{50} of ghrelin but did not affect the maximal effect of ghrelin binding or ghrelin-evoked GHSR activation, suggesting that LEAP2 acts as a competitive antagonist, as was previously shown for substance P analog and K-(D-1-Nal)-FwFwLL-NH_2 [56,70,95,96]. Interestingly, LEAP2 does not induce GHSR internalization and shows a slower dissociation rate from GHSR than ghrelin (~15 vs. ~1 minute, respectively) [54,70]. The observation that LEAP2 occupies GHSR for longer times may explain the divergent observations in the competition assays since LEAP2 added at the same time than ghrelin unmasks its competitive antagonist action, while LEAP2 added before ghrelin may act rather as a non-competitive antagonist [5,56,70]. In the absence of ghrelin, LEAP2 shifts the conformational equilibrium of GHSR away from the active state and reduces GHSR-mediated basal activation of both G_q and $G_{12/13}$ proteins pathways [54,56]. The shorter versions of LEAP2 containing the N-terminal end, including $LEAP2_{38-47}$, retain the capability to act as inverse agonists of GHSR, but their potency decrease with the reduction in their size [51]. Interestingly, LEAP2 is also able to abrogate the capability of GHSR to modulate dopamine receptor 2 signaling (unpublished

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observations). Thus, LEAP2 seems to downregulate the activity of GHSR through diverse mechanisms.

5. GHSR SIGNALING *IN VIVO*

5.1 Key features about endogenous GHSR

GHSR is mainly expressed in the pituitary gland and the brain, but also in some peripheral tissues including pancreas and cardiac muscle [2,97]. In the pituitary, GHSR is highly expressed in somatotropic cells that increase GH release in response to ghrelin [98]. Within the brain, GHSR is enriched in some brain areas with immediate access to circulating factors such as the area postrema, a sensory circumventricular organ that controls autonomic functions, and the hypothalamic arcuate nucleus, which controls food intake and some neuroendocrine axes and is located in close apposition to the median eminence, another circumventricular organ [99,100]. GHSR-expressing neurons located in the area postrema and the arcuate nucleus can sense plasma factors, including ghrelin that rapidly extravasates from the fenestrated capillaries of the area postrema and the median eminence, respectively [101–103], and presumably LEAP2, which should also passively diffuse through the fenestrated capillaries given its molecular mass. Strikingly, GHSR is also expressed in several brain areas that lack fenestrated capillaries and that, consequently, could only sense circulating GHSR ligands that are transported through either the blood-brain-barrier or the blood-cerebrospinal fluid-barrier [99,104]. Several lines of evidence in mice indicate that circulating ghrelin can be transported into the brain through the blood-cerebrospinal fluid-barrier but not through the blood-brain-barrier [99,105,106]. To the best of our knowledge, the mechanisms transporting plasma LEAP2 into the brain remain to be elucidated but the observation that LEAP2 is detected in the cerebrospinal fluid suggests that it could also cross the blood-cerebrospinal fluid-barrier (unpublished observations). In any case, ghrelin and LEAP2 present in the cerebrospinal fluid can only act on a restricted population of GHSR-expressing neurons located in periventricular brain regions. Thus, GHSR is widely distributed in several brain regions, but GHSR ligands seem to be able to act only on a small fraction of those targets.

Some *in vitro* evidence indicates that endogenous GHSR displays constitutive activity and that LEAP2 can abrogate it. In particular, LEAP2 was shown to hyperpolarize hypothalamic neurons in primary cultures [62], and $LEAP2_{38\text{-}47}$ was shown to enhance glucose-induced insulin

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release from isolated human pancreatic islets [51]. However, the magnitude of constitutive GHSR activity or the extent to which GHSR interacts with other GPCRs in *in vivo* conditions has not been directly estimated yet. The presence of GHSR in brain areas that are presumably not reached by its ligands has been hypothesized to implicate some ligand-independent actions of GHSR [104,107]. Similarly, the observation that mice lacking GHSR display more severe disturbances than mice lacking ghrelin has been considered as an indication that GHSR plays ligand-independent roles [108]. Also, the observation that the treatment of rodents with synthetic GHSR inverse agonists (such as a Substance P analog, K-(D-1-Nal)-FwFwLL-NH_2 or non-peptidic GHSR ligands based on the 1,2,4-triazole scaffold) affects food intake or body weight has been considered evidence that the constitutive GHSR activity plays physiological roles [55,95,96,109,110]. However, all these GHSR inverse agonists also reduce ghrelin-evoked GHSR activity preventing to assign, with certainty, the *in vivo* implications of the constitutive GHSR activity. The finding of short stature in people carrying a GHSR-Ala204Glu mutation, which generates a GHSR variant that lacks constitutive activity but retains ghrelin-evoked activity *in vitro*, is usually highlighted as another indication of a physiological function for the constitutive GHSR activity [83,111]. Strikingly, however, mice harboring a GHSR-Ala204Glu equivalent mutation do not increase food intake or GH plasma levels in response to ghrelin treatment, challenging the notion that clinical observations in patients carrying the mutation are exclusively due to the lack of constitutive GHSR activity [85]. Thus, physiological implications of the ligand-independent GHSR actions are yet poorly understood.

5.2 Role of ghrelin and LEAP2 as modulators of GHSR signaling in rodent models

Plasma levels of ghrelin and LEAP2 seem to fine-tune GHSR signaling according to energy balance (**Figure 4**). In *ad libitum* fed mice, ghrelin levels range ~0.2-0.5 nM, while LEAP2 levels range ~2-9 nM [5,55,62]. Thus, ghrelin levels are ~10-fold lower than LEAP2 levels and, consequently, GHSR signaling is mainly obliterated in satiated conditions [5]. Such low ghrelin/LEAP2 molar ratio may explain why mice and rats with genetic deficiency of ghrelin or GHSR lack major metabolic, neuroendocrine or behavioral alterations, as compared to wild-type animals, under satiated and non-stressful conditions [108]. Conversely, GHSR signaling is up-regulated and becomes more relevant under energy deficit conditions. For instance, ghrelin levels increase ~2.5-fold and LEAP2 levels decrease ~3-fold in fasted mice resulting in a ghrelin/LEAP2 molar ratio close to 1 [5,62]. In addition, GHSR mRNA and protein levels increase in the hypothalamus of fasted mice [109]. Overall, these changes favor an up-regulation of GHSR signaling that has physiological consequences according with the observation that GHSR-

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deficient mice, but not ghrelin-knock out mice, display a reduced fasting-induced hyperphagia and a more severe fasting-induced hypoglycemia [109,112]. One hour after re-feeding, ghrelin levels decrease and LEAP2 levels increase, restoring a ghrelin/LEAP2 molar ratio lower than 1 [5]. A critical role of GHSR signaling is also unmasked under calorie restriction, when ghrelin levels increase ~20-fold and LEAP2 levels decrease ~2-fold in mice resulting in a ghrelin/LEAP2 molar ratio higher than 1 [5]. The calorie restriction-induced up-regulation of GHSR signaling is essential for life, as indicated by the fact that several, but not all [113], studies found that mice lacking ghrelin and subjected to chronic calorie restriction fail to adequately maintain glycaemia, become lethargic and eventually die [114–116]. Notably, calorie-restricted mice overexpressing LEAP2 become moribund, indicating that not only an increase of ghrelin levels but also a decrease of LEAP2 levels are required in order to cope severe energy deficit conditions [5]. Interestingly, the observation that LEAP2 dissociates from GHSR slower than ghrelin could implicate that GHSR activity is more rapidly adjusted in a pre-prandial-to-post-prandial transition, when the ghrelin/LEAP2 molar ratio decreases, than in a satiated-to-fasted transition, when the ghrelin/LEAP2 molar ratio increases.

The role of GHSR signaling in chronic conditions of positive energy balance is less evident. Some studies found that mice with genetic deficiency of GHSR signaling, due to the lack of either ghrelin or GHSR, and fed on a high fat diet display reduced body weight and fat mass [117–119] but these observations were not recapitulated in other studies, as reviewed in [108]. In diet-induced obese mice, ghrelin levels are decreased while LEAP2 levels are increased and positively correlate with body weight and fat mass [62]. Thus, the ghrelin/LEAP2 molar ratio is reduced in obese mice, as compared to lean mice, and would result in a down-regulation of GHSR signaling that has been hypothesized to contribute to the well-established inability of obese mice to respond to exogenous ghrelin [120]. Obesity-induced alterations in plasma levels of ghrelin and LEAP2 were completely reversed by weight loss [62]. Interestingly, ghrelin levels were found elevated in anticipation to chocolate in *ad libitum* fed rats that were trained to daily receive such palatable stimulus [121]. However, plasma levels of ghrelin and LEAP2 did not differ before and after binge-like high fat diet intake in satiated mice that had time-limited access to such stimulus during 4 days [55]. Strikingly, plasma levels of ghrelin and LEAP2 in hyperphagic mice with streptozotocin-induced type I diabetes display a similar ~2-fold increase presumably due to the loss of insulin-mediated suppression of ghrelin secretion [62]. Moreover, liver gene expression and plasma levels of LEAP2 were found elevated in female mice with diet-induced steatosis, and the knockdown of liver LEAP2 in these mice resulted in an attenuation of hepatic lipogenesis and insulin resistance, suggesting that pathological elevations of LEAP2 may have

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detrimental effects [122]. Thus, the balance between ghrelin and LEAP2 levels in plasma seems to be an important signal that tunes GHSR signaling in some, but not all, metabolic conditions.

The administration of ghrelin and/or LEAP2 reveals the opposite effects of these peptides on GHSR control of food intake and glucose homeostasis. Many studies confirmed that systemic treatment with ghrelin rapidly stimulates food intake in rodents [102,123]. Also, all studies have consistently found that systemically-injected LEAP2 blocks the orexigenic effects of systemically-injected ghrelin in mice and rats [5,48,54,56]. In contrast, it is uncertain if systemically-injected LEAP2 reduces spontaneous food intake: LEAP2 treatment was initially reported to diminish 2-h food intake in mice, compared to vehicle-treated mice [5]; however, this observation could not be confirmed by others [48,51,55,56]. In addition, another study found that centrally-injected LEAP2 does not affect overnight food intake or fasting-induced hyperphagia in rats [48]. Central GHSR signaling also regulates some reward-related behaviors towards obtaining palatable foods and other rewarding stimuli [104,124,125]. In this regard, centrally-injected LEAP2 was shown to decrease high fat diet intake in satiated mice exposed to the above referred high fat diet binge-like eating protocol, suggesting that LEAP2 also abrogates reward-related effects of GHSR signaling [55]. In rodents, ghrelin treatment also increases glycaemia, likely due to the concomitant increase of the plasma levels of GH and glucocorticoids [126,127]. Systemically-injected LEAP2 has been shown to impair ghrelin-induced GH release in mice [5]. In addition, centrally-injected LEAP2 blocks the increase in glycaemia induced by central ghrelin treatment [48]. However, LEAP2 and LEAP2$_{38\text{-}47}$ do not affect glycaemia in an oral glucose tolerance test [51]. Interestingly, chronic infusion of GH was shown to partially rescue the above described deleterious effects of LEAP2 overexpression in calorie-restricted mice, suggesting that LEAP2 blocks the elevation of GH induced by increments of endogenous ghrelin [5]. Overall, studies in rodents assessing the effects of ghrelin and LEAP2 treatments have shown that some physiological impacts of GHSR signaling can be pharmacologically manipulated in a bidirectional fashion.

5.3 Role of ghrelin and LEAP2 as modulators of GHSR signaling in humans

The balance between plasma levels of ghrelin and LEAP2 in humans is just beginning to be explored. Notably, the molar ratio between ghrelin and LEAP2 levels is lower than 1 after eating in adults as well as in fasted lean adults and children, indicating that GHSR signaling is obliterated at the daily fluctuations of these ligands [62,128,129]. In adult patients with different grades of obesity, fasted LEAP2 levels positively correlate with body mass index (BMI), but only

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showed a significant ~30% increase in patients with morbid obesity [62]. As expected, fasted ghrelin levels decrease with BMI in patients with different grades of obesity, and, consequently, the ghrelin/LEAP2 molar ratio shows a stronger correlation than plasma levels of ghrelin or LEAP2 with some parameters associated with adverse consequences of obesity, such as BMI, body fat, fasting glycaemia and insulin resistance indexes [62]. In contrast to adults, LEAP2 levels were ~30% reduced and negatively correlated with the age-corrected BMI in children with overweight and obesity, while ghrelin levels and the ghrelin/LEAP2 molar ratio remained unchanged between groups [128]. Another study found that plasma levels of ghrelin and LEAP2 were similar in prepubertal children with obesity as compared to children with normal weight [129]. The same study also reported that plasma LEAP2 levels, but not ghrelin levels, were higher in girls than in boys, independently of the age-corrected BMI, and that LEAP2 levels increased after puberty only in girls suggesting a sexual dimorphism in LEAP2 levels in children and adolescents [129]. Several studies have shown that ghrelin levels display a ~40-70% post-prandial decrease in fasted lean adults eating a standard meal [130,131]. To our knowledge, two studies assessed if LEAP2 levels are affected by food intake in two cohorts of patients. One study found that: 1) plasma levels of ghrelin and LEAP2 as well as the ghrelin/LEAP2 molar ratio were unaffected 1.5 h after food intake in a cohort of previously fasted women with normal weight or obesity, 2) LEAP2 levels showed a slight ~10% increase 2 h after food intake in another set of patients with obesity, in which ghrelin levels were not assessed, and 3) post-prandial change in LEAP2 levels positively correlated with BMI in both cohorts of patients [62]. Another study found that LEAP2 levels remain unaltered in the 30-to-240 min period after food intake in patients with obesity [51]. Thus, further studies are required to clarify the impact of food intake on plasma levels of LEAP2. Plasma levels of ghrelin and LEAP2 may also be altered after bariatric surgeries. Several, but not all, studies have found that ghrelin levels decrease several months after different bariatric surgeries in patients with severe obesity, as reviewed in [132]. In terms of LEAP2, a study found that plasma LEAP2 levels show a ~10-30% decrease, which do not correlate with BMI, at ~24- or ~12-18-month after Roux-en-Y gastric bypass or vertical sleeve gastrectomy surgeries, respectively [62]. Another study found that LEAP2 gene expression increases in enteroendocrine cells of the gut immediately after Roux-en-Y gastric bypass, while fasted plasma LEAP2 levels remains unaltered 1 and 12 weeks after the surgical procedure [51]. Then, future studies are needed in order to understand ghrelin and LEAP2 plasma levels fluctuations and its impact on energy metabolism.

The balance between plasma levels of ghrelin and LEAP2 has been also assessed in some pathological conditions. Plasma levels of ghrelin and LEAP2 showed both a 45% decrease

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in women with polycystic ovary syndrome, as compared to BMI-matched healthy women [133]. In these patients, LEAP2 levels positively correlated with ghrelin levels and negatively correlated with BMI and insulin resistance indexes [133]. In patients with rheumatoid arthritis, LEAP2 levels were found ~2-fold higher, as compared to a control group, and with a positive correlation with C-reactive protein and inflammatory cytokines in plasma but not with the BMI [134]. In adults with non-alcoholic fatty liver disease, ghrelin levels were found decreased and LEAP2 levels were found increased, suggesting that the ghrelin/LEAP2 molar ratio may be associated with hepatic steatosis [122].

The effects of ghrelin treatment in humans have been extensively studied. Several, but not all, studies have found that ghrelin infusions to healthy individuals increase hunger feelings and food intake [135–137]. Also, ghrelin infusions induce some well characterized neuroendocrine (e.g. increase of GH and glucocorticoid levels), metabolic (e.g. increase of glycaemia) and autonomic (e.g. increments of gastrointestinal transit) effects [138–143]. The effects of LEAP2 treatment in humans remain to be explored. Despite its insulinotropic properties *in vitro*, a recent study found that $LEAP2_{38\text{-}47}$ failed to affect appetite, hunger sensations, resting energy expenditure, ghrelin levels, glycaemia or insulin responses in a graded glucose infusion test in humans [51]. However, this report should not discourage future studies, as $LEAP2_{38\text{-}47}$ is much less potent than LEAP2 and the pharmacokinetics parameters of the referred study (e.g. maximum $LEAP2_{38\text{-}47}$ concentration achieved) were not assessed.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The susceptibility of GHSR to be regulated by two circulating hormones with opposing actions is a very infrequent feature for a receptor. The existence of such dual regulatory system acting on GHSR suggests that the activity of this receptor is precisely regulated from inputs of the digestive system that, in turn, integrate different types of metabolic, autonomic and endocrine signals. Thus, the convergent ghrelin and LEAP2 actions on GHSR centralize a wide spectrum of information in a single molecule. Given the crucial role of GHSR on several physiological functions, it is evident that the activity of this receptor is a critical hub for neuroendocrine control. Likely, the continued research on the GHSR system will reveal further aspects of the biology of this receptor in the near future and provide a more comprehensive understanding of its physiological roles. Such knowledge will be essential for the design of drugs to modulate GHSR signaling towards the opposite ends of the biological activity spectrum. Thus, synthetic compounds that enhance GHSR actions may be useful to treat conditions associated to loss of

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appetite or weight, such as cachexia or age-related frailty. Conversely, drugs that reduce GHSR signaling may help to improve glycemic control and contribute to the treatment of patients suffering obesity or diabetes.

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AUTHOR CONTRIBUTIONS

MPC, ERM, DC, JLB, JR and MP wrote and edited the manuscript. MPC, JLB and MP designed and prepared the figures. MPC and MP devised the manuscript.

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FIGURE LEGENDS

Figure 1. Biosynthetic pathways of ghrelin and LEAP2. The figure schematically shows the putative biosynthetic steps for ghrelin (left) and for LEAP2 (right). Ghrelin and LEAP2 are produced from precursors in specific cell types and post-translationally processed in order to produce the bioactive peptides. After synthesis and storage in vesicles, ghrelin and LEAP2 are released and impact on GHSR-expressing cells.

Figure 2. GHSR, ghrelin and LEAP2 structure. **A** Crystal structure of the inactive human GHSR in complex with an antagonist molecule (PDB 6KO5). Dotted line circles I and II denote the two pockets proposed to form the ligand binding pocket of GHSR. The hydrophobic crevasse that could be responsible for binding of ghrelin octanoyl moiety is also indicated in dotted line circles with the residues colored in yellow. **B** Overlay of the best nuclear magnetic resonance (NMR) structures of the N-terminal region of ghrelin (residues 1 to 18) in its GHSR-bound state (PDB 6H3E). The octanoyl moiety is shown in red, forming a hydrophobic core with residues Phe4 and Leu5, while the dotted line circle indicates the GHSR binding domain. **C** Overlay of the best NMR structures of LEAP2 in its free state showing the conformational dynamics in the N- and C-terminal parts of the peptide (PDB 2L1Q). The disulfide bonds are shown in yellow, while the dotted line circle shows the binding domain.

Figure 3. GHSR signaling pathways modulated by ghrelin and LEAP2. GHSR displays similar binding affinity (K) for ghrelin and LEAP2, while ghrelin displays faster dissociation rates (ʋ) from GHSR than LEAP2. Blue and green arrows indicate the signaling cascades involved in GHSR ghrelin-evoked and constitutive activities, respectively. Red T-ended lines indicate the signaling pathways inhibited by LEAP2, while the question marks denote the signaling pathways that LEAP2 could affect, but experimental evidence is still missing.

Figure 4. Graphical representation of the GHSR signaling depending on the ghrelin/LEAP2 molar ratio and the energy balance state. The ghrelin/LEAP2 molar ratio is very low in satiated conditions resulting in a low GHSR signaling. Under energy deficit states, a coordinated rise in ghrelin levels and a decrease of LEAP2 levels in plasma results in an increase of the ghrelin/LEAP2 molar ratio that favors an up-regulation of GHSR signaling which, in turn, increases appetite and up-regulates hyperglycemic mechanisms. Conversely, the ghrelin/LEAP2 molar ratio decreases in animal models of obesity favoring a down-regulation of GHSR signaling in states of energy surplus (e.g., diet-induced obesity).

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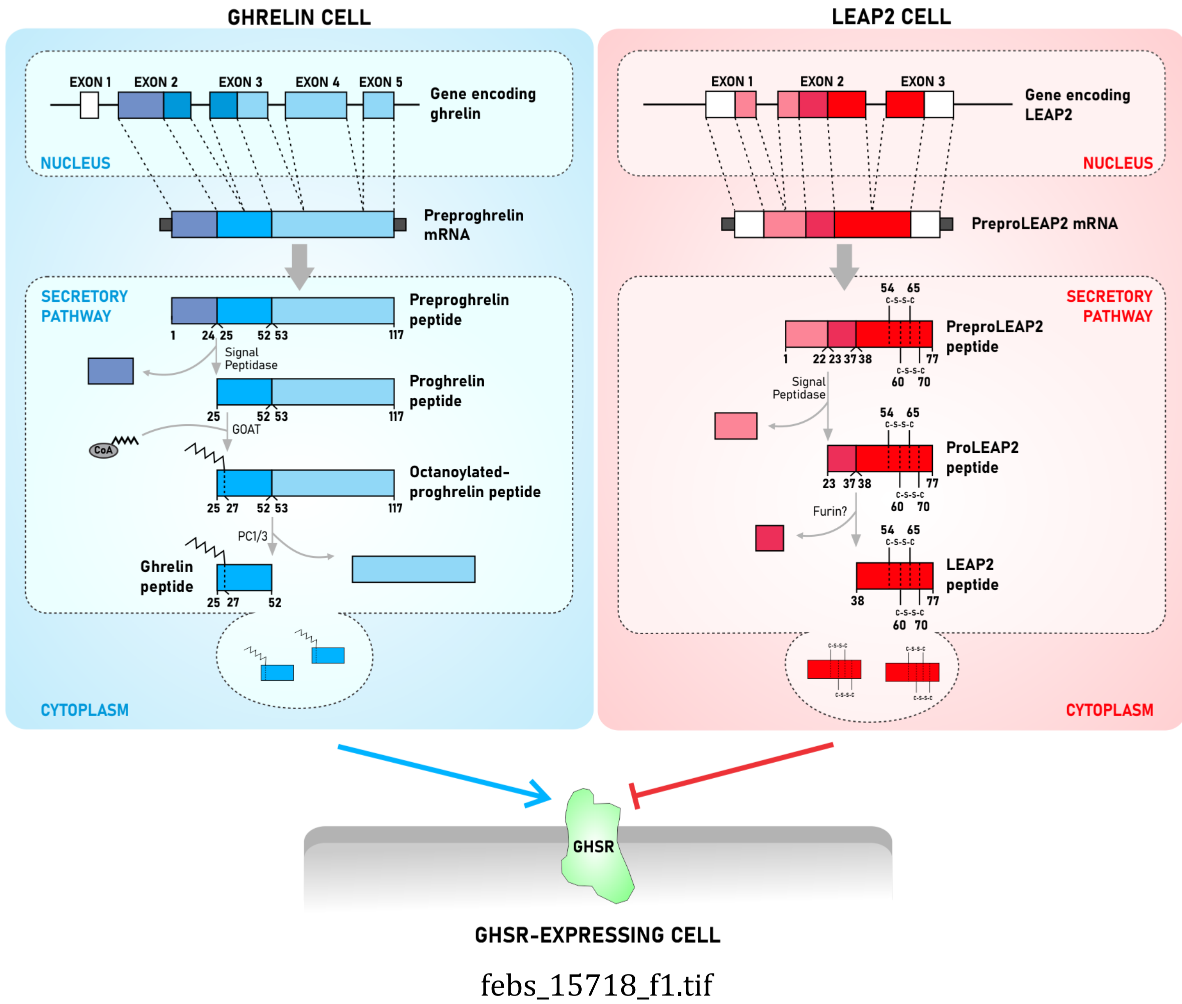

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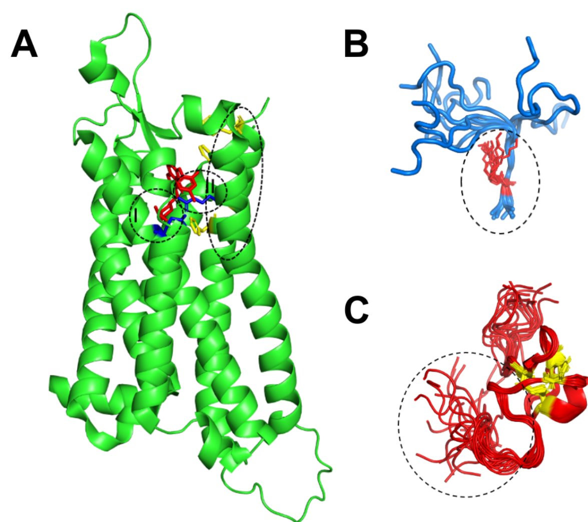

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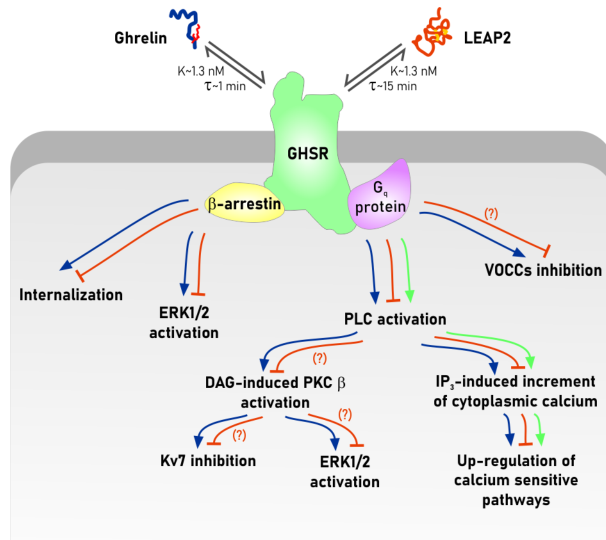

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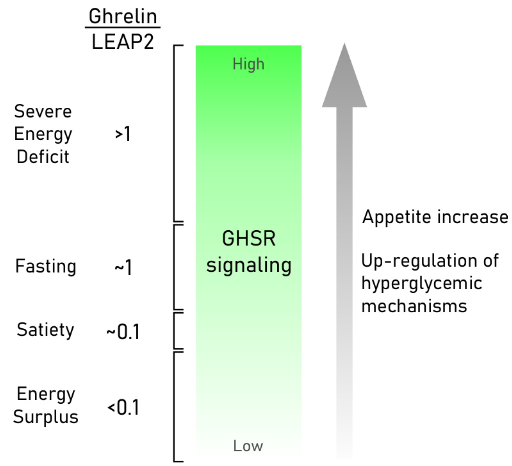

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