0163-769X/04/$20.00/0
Printed in U.S.A.

Endocrine Reviews 25(3):426–457

doi: 10.1210/er.2002-0029

Biological, Physiological, Pathophysiological, and Pharmacological Aspects of Ghrelin

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Ghrelin is a peptide predominantly produced by the stomach. Ghrelin displays strong GH-releasing activity. This activity is mediated by the activation of the so-called GH secretagogue receptor type 1a. This receptor had been shown to be specific for a family of synthetic, peptidyl and nonpeptidyl GH secretagogues. Apart from a potent GH-releasing action, ghrelin has other activities including stimulation of lactotroph and corticotroph function, influence on the pituitary gonadal axis, stimulation of appetite, control of energy balance, influence on sleep and behavior, control of gastric motility and acid secretion, and influence on pancreatic exocrine and endocrine function as well as on glucose metabolism. Cardiovascular actions and modulation of proliferation of neoplastic cells, as well as of the immune system, are other actions of ghrelin. Therefore, we consider ghrelin a gastrointestinal peptide contributing to the regulation of diverse functions of the gut-brain axis. So, there is indeed a possibility that ghrelin analogs, acting as either agonists or antagonists, might have clinical impact. (*Endocrine Reviews* 25: 426–457, 2004)

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I. Introduction

GHRELIN IS A 28-amino residue peptide predominantly produced by the stomach (Fig. 1). Substantially lower amounts were detected in bowel, pancreas, kidneys, the immune system, placenta, testes, pituitary, lung, and hypothalamus (1–12). Ghrelin displays strong GH-releasing activity, which is mediated by the activation of the so-called GH secretagogue (GHS) receptor type 1a (GHS-R 1a) (13). Before the discovery of ghrelin, this orphan receptor had been shown to be specific for a family of synthetic, peptidyl and nonpeptidyl GHS (1, 14–23). GHS-Rs are concentrated in the hypothalamus-pituitary unit but are also distributed in other central and peripheral tissues (8, 13, 14, 20, 22–30). Indeed, apart from stimulating GH secretion, ghrelin and many synthetic GHS (Fig. 2): 1) exhibit hypothalamic activities that result in stimulation of prolactin (PRL) and ACTH secretion; 2) negatively influence the pituitary-gonadal axis at both the central and peripheral level; 3) stimulate appetite and a positive energy balance; 4) influence sleep and behavior; 5) control gastric motility and acid secretion; and 6) modulate pancreatic exocrine and endocrine function and affect glucose levels.

Cardiovascular actions and modulation of the proliferation of neoplastic cells, as well as of the immune system, are also actions of ghrelin and/or other GHS (2, 8, 9, 14, 16, 20, 25, 27, 31–58). Given this wide spectrum of biological activities, it is evident that the discovery of ghrelin opened many new perspectives within neuroendocrine and metabolic research and even has an influence on fields of internal medicine such as gastroenterology, immunology, oncology, and cardiology. It is therefore increasingly likely that ghrelin and its GHS analogs, acting as either agonists or antagonists on different physiological and pathophysiological processes, might have clinical impact and therapeutic potential.

Abbreviations: AGRP, Agouti-related protein; CNS, central nervous system; CST, cortistatin; GABA, γ-aminobutyric acid; GHD, GH deficiency or GH-deficient; GHRP, GH-releasing peptide; GHS, GH secretagogue(s); GHS-R 1a, GHS receptor type 1a; GLP, glucagon-like peptide; HDL, high-density lipoprotein; NPY, neuropeptide Y; POMC, proopiomelanocortin; PRL, prolactin; PWS, Prader-Willi syndrome; SS, somatostatin.

Endocrine Reviews **is published bimonthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

II. Historical Background

The gastric hormone ghrelin was identified as an endogenous ligand for the former orphan receptor GHS-R 1a (1, 13, 14); the discovery of this receptor followed by 20 yr that of synthetic GHS, which specifically binds it (1, 14, 17, 19–21,

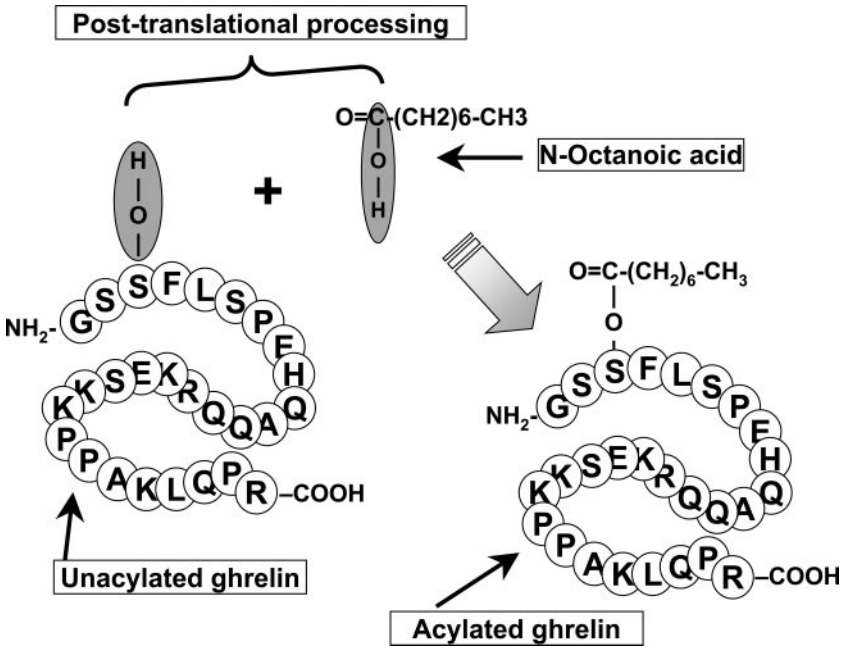

FIG. 1. Ghrelin is the only known natural peptide in mammalian biology in which acylation of one amino residue is required for at least the majority of its biological activities. Under the influence of a still unknown acyl-transferase, a hydroxyl group of serine at position 3 of the ghrelin molecule is octanoylated. This posttranslational modification of ghrelin is essential for binding and activation of the GHS-R 1a, for the GH-releasing capacity of ghrelin, and most likely also for its action on endocrine axis, energy balance, and glucose homeostasis. Several naturally occurring variants of ghrelin have been reported based on the acylation at the serine-3 position, including nonacylated, octanoylated (C8:0), decanoylated (C10:0), and possibly decenoylated (C10:1) ghrelin. Any other synthetic variant of ghrelin with a chemical modification of either the acyl group or the N-terminal amino residue sequence did not activate or bind the receptor GHS-R 1a. However, ghrelin did still bind and activate the GHS-R 1a *in vitro* after modification or even significant deletion of C-terminal amino residues. It however remains unclear whether the same modalities are relevant *in vivo*. Although the major active form of human ghrelin is a 28-amino acid peptide with an octanoylation at the serine-3 position, the vast majority (~80–90%) of circulating ghrelin has been found to be nonacylated. This predominant form of serum ghrelin seems to be devoid of any effects on endocrine axes or energy balance, as previously expected based on its inability to bind and activate GHS-R 1a, which is still the only identified ghrelin receptor. However, nonacylated ghrelin does have cardiovascular and antiproliferative effects, and it seems tempting to speculate that these activities are mediated by yet to be identified receptor families or subtypes (1). In the absence of further information on the tissue specificity, reversibility, balance, and enzyme kinetics of the (des-) octanoylation process, the information one can possibly gain from plasma ghrelin quantification is very limited but should include the analysis of both total and acylated ghrelin (17, 131–133).

28, 59–72). This makes the discovery of ghrelin an example of reverse pharmacology, which in this case means that it started with the synthesis of analogs and it ended with the discovery of a natural ligand via the discovery of a natural receptor.

Synthetic GHS are a family of ligands, including peptidyl and nonpeptidyl molecules (Table 1) (8, 14, 20, 68, 69, 73–82). The first synthesized molecules were nonnatural peptides [GH-releasing peptides GHRPs)] that were designed by Bowers and Momany (59, 60, 68, 73) in the late 1970s. They were metenkephalin derivatives devoid of any opioid activity (Table 2).

GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) was the first hexapeptide to actively release GH *in vivo*, in humans even more than in animals. One of its most remarkable properties was that GHRP-6 showed strong GH-releasing activity even after oral administration, although with low bioavailability and short-lasting effects (14, 68, 73, 74, 83). Further research that aimed to select orally active molecules with better bioavailability and longer half-lives led to the synthesis of other GHRPs as well as the discovery of orally active nonpeptidyl molecules. The most representative of these nonpeptidyl GHS that was studied in humans was the spiroindoline L-163,191 (MK-0677) (14, 84–105). MK-0677 has been shown to possess a high bioavailability and is able to enhance 24-h GH secretion after a single oral administration (14, 84–105). All these data explain why MK-0677 became the most likely candidate compound for drug treatment of GH deficiency (GHD) in childhood. Also, it was suggested that, as an orally active anabolic drug, it might play a therapeutic role for antiaging purposes in frail elderly subjects (14, 85, 86, 88–90, 92, 96, 97, 99, 102–105).

Very recently, another new peptidomimetic GHS with potent and selective GH-releasing activity was synthesized and called EP1572 UMV1843 [Aib-D-Trp-DgTrp-CHO]). EP1572 shows binding potency to the GHS-R in animal and human tissues similar to that of ghrelin and peptidyl GHS and induces marked GH increase after sc administration in neonatal rats. Preliminary human data show that acute iv EP1572 administration (1.0 μg/kg) induces strong and selective increases in GH levels. Moreover, a single oral EP1572 administration strongly and reproducibly increases GH levels even after a dose as low as 0.06 mg/kg (106).

MK-0677 resulted in the discovery and cloning of the GHS-R. The existence of this GHS-R, as shown by mRNA expression, had been indicated already by binding studies

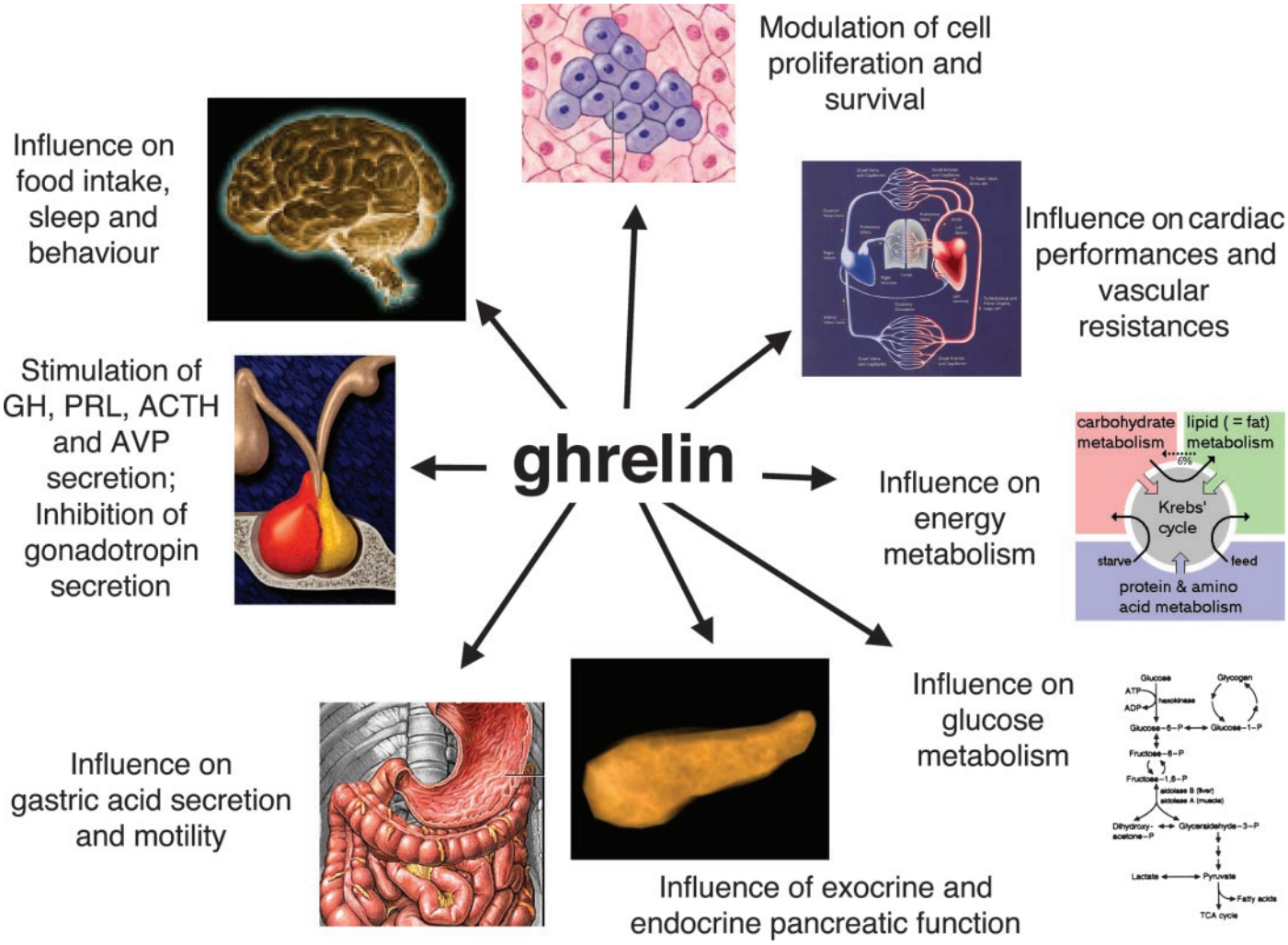

FIG. 2. Known biological activities of ghrelin. Some of the effects of ghrelin shown here are believed to be indirectly mediated via pituitary hormones or hypothalamic neurocircuits and their efferent pathways; others, such as the effect on the cardiovascular system, appear to be direct. Depending on the origin of the hormone, which is mainly derived from the stomach but also expressed in the pancreas, the hypothalamus, the pituitary, the duodenum, and other organs, these effects may have endocrine, paracrine, or autocrine character. AVP, Arginine vasopressin.

TABLE 1. Primary structure of ghrelin from domesticated species aligned to human ghrelin

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Species
G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Human
G	S	S	F	L	S	P	E	H	Q	***K***	***T***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Gerbil
G	S	S	F	L	S	P	E	H	Q	***K***	***A***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Mouse
G	S	S	F	L	S	P	E	H	Q	***K***	***A***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Rat
G	S	S	F	L	S	P	E	H	Q	***K***	***L***	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Dog
G	S	S	F	L	S	P	E	H	Q	***K***	V	Q	Q	R	K	E	S	K	K	P	***A***	A	K	L	***K***	P	R	Pig
G	S	S	F	L	S	P	E	H	Q	***K***	***L***	□	Q	R	K	E	***A***	K	K	P	***S***	***G***	***R***	L	***K***	P	R	Cattle
G	S	S	F	L	S	P	E	H	Q	***K***	***L***	□	Q	R	K	E	***P***	K	K	P	***S***	***G***	***R***	L	***K***	P	R	Sheep
G	S	S	F	L	S	P	***T***	***Y***	***K***	***N***	***I***	Q	Q	***Q***	K	***D***	***T***	***R***	K	P	***T***	A	***R***	L	***H***	***R***	R	Chicken
G	S	S	F	L	S	P	***S***	□	Q	R	□	***P***	Q	***G***	K	***D***	□	K	K	P	P	□	***R***	***V***	***G***	***R***	R	Eel
G	S	S	F	L	S	P	***S***	□	□	□	□	□	Q	***K***	***P***	***Q***	***N***	K	***V***	***K***	***S***	***S***	***R***	***I***	***G***	***R***	***Q***	Tilapia

Bold and *italic* indicates that residue is different from that in human; □ indicates space added for alignment to the human ghrelin primary structure.

(14, 24, 26, 29, 30, 47, 73, 107–119). Studies focusing on the distribution of the identified GHS-Rs showed a particular concentration of these receptors in the hypothalamus-pituitary area. However, GHS-R expression and/or the presence of specific binding sites has been found in other brain areas and peripheral, endocrine, and nonendocrine animal and human tissues (1–9, 22, 24–26, 30, 120, 121). Actually, both the concentration of binding sites and the displacement of peptide-radioligand by ghrelin suggest that the majority of binding in peripheral tissues is not specific for ghrelin or MK-

TABLE 2. Studies of peptidyl and nonpeptidyl GHS

Year	Peptidyl GHS (GHRPs)	Refs.	Nonpeptidyl GHS	Refs.
1977	(D-Trp^2)-metenkephalin	59, 60, 68, 73		
1984	GHRP-6	14, 68, 73, 74, 83		
1991	GHRP-1	270, 305		
1992			L-692,429	103–105, 256, 257, 261, 468, 469
1993	GHRP-2	263, 470–474		
1994	Hexarelin	303, 475–478	L-692,585	272, 285, 479
1995			MK-0677	14, 84–105
1996	EP-51389	167, 480, 481		
1998	Ipamorelin	76, 482		
1999			NN-703	483–485
2000			CP-424,391	75
2001			SM-130686	486
2002			EP-01572	106

0677. The studies using labeled GHRPs overestimate the concentration of specific ghrelin binding because they exhibit high capacity and low affinity binding in addition to limited capacity ghrelin-specific binding. Anyway, hypothalamopituitary and peripheral distribution of GHS-Rs probably explains both the GH-releasing effect of GHS and their other endocrine and nonendocrine biological activities (2, 8, 9, 14, 16, 20, 25, 27, 31–56, 122).

As discovered by Kojima *et al*. (1) in 1999, ghrelin appeared to be a 28-residue peptide that is predominantly produced by the stomach but is also expressed in many other tissues (1–9, 12, 123). Ghrelin is produced in the stomach by the enteroendocrine X/A-like cells that represent a major endocrine population in the oxyntic mucosa. The hormonal product of these cells had not been previously clarified (1, 3, 124–127). Ghrelin production has also been reported in neoplastic tissues as gastric and intestinal carcinoids (126, 128) and in medullary thyroid carcinomas (129).

Ghrelin is the first natural hormone to be identified in which the hydroxyl group of one of its serine residues is acylated by n-octanoic acid (1). This acylation is essential for binding to the GHS-R 1a, for the GH-releasing capacity of ghrelin, and most likely for its other endocrine actions also (1, 17, 21, 130) (Fig. 1). Nonacylated ghrelin, which is present in human serum in far greater quantities than acylated ghrelin, seems to be devoid of any endocrine action. However, it is able to exert some nonendocrine actions including cardiovascular and antiproliferative effects, probably by binding different GHS-R subtypes or receptor families (3, 27).

There is another endogenous ligand for the GHS-R 1a that can be isolated from the endocrine mucosa of the stomach. Des-Gln14-ghrelin has undergone the same process of acylation at its Ser3 residue and is homologous to ghrelin except that it lacks one glutamine. Des-Gln14-ghrelin is the result of alternative splicing of the ghrelin gene, and it seems to possess the same hormonal activities as ghrelin (1, 131). Studies with several analogs of ghrelin with various aliphatic or aromatic groups in the side chain of residue 3 and several short peptides derived from ghrelin as well showed that bulky hydrophobic groups in the side chain of residue 3 are essential for maximum agonist activity. In addition, short peptides encompassing the first four or five residues of ghrelin were found to functionally activate GHS-R 1a about as efficiently as the full-length ghrelin. Thus, the entire sequence of ghrelin is not necessary for activity; the Gly-Ser-Ser(n-octanoyl)-Phe segment appears to constitute the active core required for agonist potency at GHS-R 1a (17, 132). Hosoda *et al*. (133) isolated, in the course of purification of ghrelin from the stomach, human ghrelin of the expected size, as well as several other ghrelin-derived molecules that could be classified into four groups by the type of acylation observed at the serine-3 position. These peptides were found to be nonacylated, octanoylated (C8:0), decanoylated (C10:0), and possibly decenoylated (C10:1). The major active form of human ghrelin is a 28-amino acid peptide octanoylated at the serine-3 position, as was found for rat ghrelin. Both ghrelin and the ghrelin-derived molecules were found to be present in plasma as well as stomach tissue. Del Rincon *et al*. (134) pointed out that identification and characterization of the novel gastric peptide hormone, named motilin-related peptide by Tomasetto *et al.* (127, 135), were strictly connected to ghrelin. Motilin-related peptide shows the same amino acid sequence as ghrelin, reflecting that the same gene encoding for this peptide was discovered by two different groups and was given two different names.

That the scientific work on the GHS also led to the discovery of the motilin receptor is not by chance; the motilin receptor is a member of the GHS-R family having a 52% identity (112, 117, 118, 127, 134–139). This former orphan G protein-coupled receptor was isolated based on its high homology with the GHS-R, and through ligand screening assays motilin was identified as its endogenous ligand. However, unlike ghrelin, acylation of motilin is not needed for activation of its receptor (112). Prepromotilin, which is also produced by the enteroendocrine cells of the stomach, is almost identical with human preproghrelin, except for the serine-26 residue that is not octanoylated in the prepromotilin-related peptide; however, human ghrelin and motilin show only 36% homology (1, 112, 117, 127, 134–140). Motilin and motilin receptors have been well characterized in humans and dogs, whereas rodents do not have a motilin receptor. The ability of motilin to exert some stimulatory effect on GH secretion and some orexigenic effect after central administration cannot be mediated by the GHS-R 1a, because motilin does not activate GHS-R 1a (112, 137, 140, 141). On the other hand, ghrelin does not activate motilin receptors (142). Therefore, we consider both ghrelin and motilin as representatives of a novel family of gastrointestinal peptides contributing to the regulation of diverse functions of the gut-brain axis. This in itself is a remarkable turn of a story

that started as a field of research focused on GH secretion alone.

III. The Biology of a Ubiquitously Expressed Hormone and Its Receptor Family

A. Known and unknown GH secretagogue receptors

In addition to the physiological stimulation by hypothalamic GHRH, the release of GH from the pituitary is stimulated by small synthetic peptidyl and nonpeptidyl molecules called "GH secretagogues" (for reviews, see Refs. 48 and 74). They act through a specific G protein-coupled receptor (13), the GHS-R, for which the ligand was unknown until a Japanese group of scientists led by M. Kojima (1) isolated an endogenous ligand specific for the GHS-R, ghrelin, from the stomach. The discovery of this novel gastric hormone, ghrelin, which consists of 28 residues containing an n-octanoyl modification at serine 3, has been recently reviewed by Bowers (70), Kojima *et al*. (143), Inui (52), and Muccioli *et al*. (8).

The GHS-R is expressed by a single gene found at human chromosomal location 3q26.2 (113, 114). Two types of GHS-R cDNAs, which are presumably the result of alternate processing of a pre-mRNA, have been identified and designated receptors 1a and 1b (13, 14, 114) (for reviews, see Refs. 112, 144, 145; also see Ref. 146). Their sequences do not show significant homology with other known receptors; the closest receptor relatives are the neurotensin with 34.9% identity and motilin 1a with 51.6% identity (113). cDNA 1a encodes a receptor, named GHS-R 1a, of 366 amino acids with seven-transmembrane regions and a molecular mass of approximately 41 kDa. The 1b cDNA encodes a shorter form, named the GHS-R 1b, which consists of 289 amino acids with only five-transmembrane regions (13). GHS-R 1b is derived by readthrough of the intron, which produces an in-frame stop codon so that the potential translation product has an identical N terminus with transmembrane domains 1–5 but lacks transmembranes 6 and 7. Although this process does not seem to end in the transcription of a protein, GHS-R 1b expression, however, is widespread in many endocrine and nonendocrine tissues, but its significance remains to be determined (13, 22, 112).

The human GHS-R 1a shares 96 and 93% identity with the rat and pig GHS-R 1a, respectively, and the existence of this receptor can apparently be extended to pre-Cambrian times because amino acid sequences strongly related to human GHS-R 1a have been identified in teleost fish (117). These observations strongly suggest that the GHS-R 1a is highly conserved across the species and probably does have an essential biological function.

The binding of ghrelin and synthetic GHS (such as the peptidyl GHRP-6 and the nonpeptidyl derivative MK-0677) to the GHS-R 1a activates the phospholipase C signaling pathway, leading to increased inositol phosphate turnover and protein kinase C activation, followed by the release of Ca^{2+} from intracellular stores (14, 147). GHS-R activation also leads to an inhibition of K^+ channels, allowing the entry of Ca^{2+} through voltage-gated L-type, but not T-type channels (148, 149). Unlike the GHS-R 1a, the GHS-R 1b failed to bind GHS and to respond to GHS (13), and its functional role remains to be defined. Synthetic GHS and ghrelin, as well as des-Gln14-ghrelin, a natural isoform that has the same GH-releasing activity as ghrelin (131), bind with high affinity to the GHS-R 1a; their efficacy in displacing [^{35}S]MK-0677 or [^{125}I][Tyr^4]ghrelin binding to pituitary membranes correlates well with concentrations required to stimulate GH release (14, 21, 150). The n-octanoyl group at serine 3 of the ghrelin molecule seems to be essential for the binding and bioactivity of the hormone, at least in terms of GH release. In fact, the nonacylated ghrelin, which circulates in amounts far greater than the acylated form (131), does not displace radiolabeled ghrelin from its hypothalamic or pituitary binding sites (21) and has no GH-releasing or other endocrine activities in rat (1, 70). In man also, the administration of nonacylated ghrelin does not induce any change in the hormonal parameters or in glucose levels, indicating that at least in humans nonacylated ghrelin does not possess endocrine activities of human acylated ghrelin (151).

Interestingly, it has been reported that ghrelin binds a species of high-density lipoprotein (HDL) associated with the plasma esterase, paraoxonase, and clusterin. Both free ghrelin and paraoxon, a substrate for paraoxonase, can inhibit this interaction. Some endogenous ghrelin is found to copurify with HDL during density gradient centrifugation. This interaction links the orexigenic peptide hormone ghrelin to lipid transport and a plasma enzyme that breaks down oxidized lipids in low-density lipoprotein (see Fig. 4). Furthermore, the interaction of the esterified ghrelin with a species containing an esterase suggests a possible mechanism for the conversion of ghrelin to des-acyl ghrelin (152).

Recent studies, dealing with the minimal sequence of ghrelin needed to activate the GHS-R 1a, have shown in HEK-293 cells transfected with the human GHS-R 1a that short octanoylated peptides encompassing the first four to five residues of ghrelin were capable of increasing intracellular Ca^{2+} almost as efficiently as the full-length ghrelin (17, 130). Based on these *in vitro* results, it has been postulated that the active core required for the activation of the receptor is the Gly-Ser-Ser(n-octanoyl)-Phe sequence. Indeed, the amino-terminal 7 residues of ghrelin are conserved between species (Table 1). However, the ability of the above ghrelin derivatives to activate the GHS-R 1a in transfected cells does not seem indicative of their capability to stimulate GH secretion from somatotroph cells. In fact, we have recently demonstrated that octanoylated ghrelin-(1–4) or octanoylated ghrelin-(1–8) is unable to stimulate GH release in rats, and neither of these two truncated molecular forms of ghrelin is effective in displacing [^{125}I][Tyr^4]ghrelin from its binding sites in membrane preparations from human hypothalamus or pituitary gland (153). Possibly, overexpression of the GHS-R 1a or lack of the other receptor populations physiologically present in pituitary cells may be responsible for the reported activity of ghrelin analogs in HEK-293 cells (17, 130). Other study groups working on the same cells expressing human or pig GHS-R 1a have found that adenosine also activates the transfected receptor but, similar to short ghrelin analogs, does not possess a biological counterpart able to stimulate GH secretion and amplify the GHRH effects on normal pituitary cell cultures (154). It has been suggested that

adenosine is a partial agonist of the GHS-R 1a and binds to a receptor site distinct from the binding pocket recognized by MK-0677 and GHRP-6 (155). More recently, we have reported (19) that the GHS-R is also bound by another endogenous molecule such as cortistatin (CST), a neuropeptide homologous to somatostatin (SS), which itself is unable to recognize the GHS-R 1a. This finding supports the hypothesis that natural ligands other than ghrelin or adenosine might modulate the activity of the GHS-R.

Expression of the GHS-R 1a was shown in the hypothalamus and anterior pituitary gland (13, 30, 156, 157), consistent with its role in regulating GH release. The GHS-R 1a is largely confined to somatotroph pituitary cells and to the arcuate nucleus (13, 14, 158), a hypothalamic area that is crucial for the neuroendocrine and appetite-stimulating activities of ghrelin and synthetic GHS (120, 159). This is supported by the demonstration that ghrelin, as well as synthetic GHS, effectively stimulates the expression of some markers of neural activity (c-*fos* and early growth response factor-1) in arcuate nucleus neurons (160, 161). The activated hypothalamic cells include GHRH-containing neurons, but also cells expressing the appetite-stimulating neuropeptide Y (NPY) (71, 158) and the endogenous melanocortin receptor inverse agonist, agouti-related protein (AGRP) (31). Detectable levels of GHS-R 1a mRNA were also demonstrated in various extrahypothalamic areas such as the dentate gyrus of the hippocampal formation, CA2 and CA3 regions of the hippocampus, the pars compacta of the substantia nigra, the ventral tegmental area and dorsal and medial raphe nuclei and Edinger-Westphal nucleus, pons and medulla oblongata (24, 30, 162), possibly indicating its involvement in as yet undefined extrahypothalamic actions. More recent localization studies have demonstrated GHS-R expression in multiple peripheral organs, although the RT-PCR primers were generally not selected to differentiate GHS-R 1a from 1b (1–5, 7–9, 22, 24–26, 30, 120, 121, 163). mRNA was shown in the stomach and intestine (3), pancreas (30), kidney (4), heart and aorta (38, 164, 165), as well as in different human pituitary adenomas (6, 163) and various endocrine neoplasms of lung (166), stomach (126), and pancreas (7, 121, 163). These data are in accord with the reported observations indicating, for ghrelin and synthetic GHS, broader functions beyond the control of GH release and food intake (see *Section V.C*).

Ghrelin and GHS compounds exhibit a high binding affinity to the cloned GHS-R 1a. However, there is evidence, indicating that there are additional binding sites for GHS. Specific binding sites for Tyr-Ala-hexarelin [Tyr-Ala-His-D-2Methyl-Trp-Ala-Trp-D-Phe-Lys-NH_2], other peptidyl GHS (GHRP-2 [D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2], GHRP-6, and hexarelin [His-D-2Methyl-Trp-Ala-Trp-D-Phe-Lys-NH_2]) with a receptor density that at least equals the density that was found in the pituitary, have been found in rat and human heart (24, 25, 107, 167, 168), as well as in a wide range of other nonendocrine peripheral human tissues such as lung, arteries, skeletal muscle, kidney, and liver (20, 26). These binding sites are presumably not ghrelin receptors, because they show a very low binding affinity for ghrelin (26). As reported by Bodart *et al.* (167), the cardiac GHS-R has a molecular mass larger (84 kDa) than that of GHS-R 1a and shows no homology with this receptor. The predicted amino acid sequence of the GHS-R expressed in heart muscle is similar to that of CD36, a multifunctional receptor also known as glycoprotein IV (49).

The functional significance of receptors for peptidyl GHS in peripheral nonendocrine tissues is still unknown. Some findings in the cardiovascular system suggest that these binding sites could mediate GH-independent cardioprotective activities exerted by peptidyl GHS, but not by ghrelin (see *Section V.C*).

Recently, we have found ghrelin receptors with a binding profile different from the GHS-R 1a ghrelin receptor in human thyroid and breast tumors, as well as in related cancer cell lines (27, 169). In fact, binding of acylated ghrelin to these receptors is surprisingly inhibited by nonacylated ghrelin, as well as by some synthetic GHS (27, 169); a receptor with the same binding profile has been demonstrated at the cardiovascular level (8, 170). It has to be emphasized that nonacylated ghrelin, although unable to bind to the classical GHS-R 1a and to show any endocrine activity, exerts antiproliferative (27) and cardioprotective effects (171). This is illustrated in the experiment of Fig. 1 that compares the ability of unlabeled ghrelin and nonacylated ghrelin to displace [^{125}I][Tyr^4]ghrelin binding to membranes from cultured pituitary explants, H9C2 cardiomyocytes, and MCF-7 mammary carcinoma cells.

B. Known and unknown ligands of the GH secretagogue receptors

Taking into account the GHS-R 1a as receptor of reference, acylated ghrelin and Des-Gln14-ghrelin are its natural ligands; in fact, both molecules possess the same endocrine activities (150).

There are also other natural ligands of the GHS-R 1a. Besides adenosine that binds and activates the receptor (112, 154, 155), it has been demonstrated that CST, a neuropeptide, binds with high affinity the GHS-R 1a in human hypothalamus and pituitary tissues (19, 112, 154, 172). CST is a recently described neuropeptide showing high structural homology with SS (173–188) that binds to all SS receptor subtypes with an affinity (1–2 nM) close to that of SS (103, 189, 190). In fact, in humans as well as in animals, CST and SS exhibit the same endocrine activities (191, 192). The existence of specific receptors that selectively bind SS or CST has been hypothesized (189, 190), based on evidence that CST possesses an action profile different from SS (189, 193, 194) and on the fact that CST and SS are often coexpressed in the same neurons but are regulated by different stimuli (189, 195, 196). Given these findings, the ability of CST to bind the GHS-R 1a is of particular relevance because SS and its fragments do not bind the same receptor (19, 112, 154, 172). Interestingly, the classical synthetic SS analogs, *i.e.*, octreotide, lanreotide, and vapreotide, bind the GHS-R 1a with an affinity lower than that of CST (19, 112, 154, 172). These findings have generated the hypothesis that CST could play a potential role in the control of somatotroph secretion via both SS and GHS-Rs. Where this is the case, CST would represent the link between ghrelin and the SS/CST system that had not previously been demonstrated.

On the other hand, the GHS-R 1a is unlikely to be the only

GHS-R (see *Section III. A*). It has already been demonstrated that a GHS-R subtype able to bind nonacylated as well as acylated ghrelin exists and likely mediates biological activities (27). This report might provide another explanation besides the existence of different pockets within the GHS-R 1a to explain the observation that different molecules are able to bind, but not activate it (112).

Another GHS-R subtype likely mediates the influence of ghrelin on insulin secretion and glucose metabolism, because this effect is not shared by synthetic peptidyl GHS that generally mimic ghrelin actions (35). The cardiovascular receptor that only binds peptidyl GHS is unlikely to be a GHS-R, because it does not bind ghrelin (25, 26, 49, 49, 167, 197).

Given this complexity, it is clear that further studies are required to clarify whether ghrelin is the sole ligand or one of a number of ligands activating the GHS-R 1a and whether that receptor used for ghrelin isolation is the sole receptor or one of a group of receptors for such ligands.

IV. Control of Ghrelin Secretion: Indications for Its Importance in Biology

Although the majority of circulating ghrelin originates from the stomach and the small bowel (3, 124), ghrelin is expressed in a variety of tissues that include the stomach, the intestine, the pituitary, the placenta, lymphocytes, the testes, the lungs, the kidney, the pancreas, and the hypothalamus (1–12).

The activation level of the ghrelin receptor(s) is the parameter that is decisive for ghrelin action. Regulation of the extent and magnitude of ghrelin action therefore involves several mechanisms that are, at least in part, independent. These mechanisms include: 1) the regulation of transcription and translation of the ghrelin gene; 2) the level of enzymatic activity of the putative acyl transferase that is responsible for the posttranslational octanoylation of the ghrelin molecule; 3) secretion rates of the bioactive ghrelin molecule; 4) putative enzymatic processes deactivating circulating ghrelin; 5) possible influence of ghrelin binding proteins on the hormone's bioactivity; 6) variable accessibility of target tissue (*i.e.*, blood-brain barrier transport); 7) clearance or degradation of ghrelin by kidney or liver passage; 8) circulating concentration of additional endogenous ligands or other possibly cross-reacting hormones; 9) the amount of expression of ghrelin receptor(s) in target tissue; and 10) their sensitivity to the level of intracellular signaling mechanisms. Most studies to date have focused on changes in gastric ghrelin mRNA expression or variation of circulating ghrelin concentration as quantified by immunoassay measurements from plasma samples. The search for a ghrelin activating acyltransferase, for possible additional endogenous ligands to ghrelin receptor(s), and for specific ghrelin binding proteins is still ongoing. However, a few studies have started to shed light on important issues such as blood-brain barrier transport of ghrelin (198) and the regulation of ghrelin receptor expression, *i.e.*, in the hypothalamus (199). Analysis of the regulation of ghrelin expression levels in tissues other than stomach is hardly possible due to the small amounts of peptide present in these organs.

The measurement of ghrelin immunoreactivity involves technical difficulties, which imply that all results based on the concentration of circulating ghrelin as quantified by immunoassays should still be interpreted with much caution. Although commercially available ghrelin immunoassays are very likely to reflect total ghrelin peptide concentration, reliable methods to routinely quantify individual ghrelin species are still not available. An ideal tool would be a sensitive and specific sandwich immunoassay based on two monoclonal antibodies recognizing an epitope associated with the octanoyl side chain and another one at the C-terminal end of the 28-amino residue peptide. Existing assays targeting the C-terminal end of the molecule miss potential crucial changes in the percentage of circulating octanoylated ghrelin. Immunoassays targeting the octanoyl side chain of the molecule might suffer from interference from other octanoylated molecules, which are likely to exist. Therefore, plasma ghrelin levels as described by several research groups vary according to the antiserum used and are influenced by varying techniques, such as the use of an additional extraction step. Furthermore, there are controversial data on the stability of ghrelin in plasma samples, the influence of storing time and thaw/freeze cycles, pH changes, or the necessity for enzyme-blocking additives to plasma samples before measuring ghrelin. Although absolute plasma ghrelin levels and ghrelin reference standards still have to be determined, it appears reasonable to investigate ghrelin regulation and physiology by measurement of relative differences of circulating total ghrelin levels using available immunoassays. In the following section, existing knowledge regarding the regulation of ghrelin expression and secretion is summarized, although this current model might have to be revised substantially when details of the unknown mechanisms and open questions mentioned above become available.

Ghrelin mRNA expression as well as ghrelin peptide have been localized most impressively in the oxyntic glands, specifically the X/A-like cells of the gastrointestinal tract. These cells represent about one fourth of all endocrine cells in the oxyntic mucosa, whereas other cells within these glands, such as histamine-rich enterochromaffin-like cells (~70%) and D-(SS) cells (10%), are not ghrelin positive (3, 124). Ghrelin is found from the stomach to the colon with caudally decreasing density of expression, which is in agreement with the fact that X/A-like cells are not strictly confined to oxyntic mucosa (3, 124). Ghrelin-containing enteroendocrine cells mostly have no continuity with the lumen, probably respond to physical and/or chemical stimuli from the basolateral side, and are closely associated with the capillary network running through the lamina propria (3, 124). A recent study shows that ghrelin-secreting cells occur as open- and closed-type cells (open or closed toward the lumen) with the number of open-type cells gradually increasing in the direction from the stomach to the lower gastrointestinal tract (200).

Although a classical endocrine role for ghrelin as a peptide hormone that is secreted into this capillary network is evident, local paracrine activities of ghrelin might play an additional role (3, 124). Removal of the stomach or the acid-producing part of the stomach in rats reduces serum ghrelin concentration by approximately 80%, further supporting the view that the stomach is the main source of this endogenous

GHS-R ligand (3, 124). However, in a recent study, plasma levels of ghrelin after total gastrectomy gradually increased again (133), suggesting that the stomach is the major source of circulating ghrelin but that other tissues can compensate for the loss of ghrelin production after gastrectomy (201). Cummings and co-workers (202–204) showed that total plasma ghrelin is hardly detectable after gastric bypass surgery, a phenomenon that is interpreted as a shutdown of gastric ghrelin-secreting cells due to a lack of contact with ingested nutrients. On the other hand, no evidence for Roux-en-Y gastric bypass surgery *per se* having an effect on ghrelin levels, independent of weight loss, was obtained (205).

Small concentrations of ghrelin are found in the pancreas (206), where ghrelin immunoreactivity was localized in a subgroup of endocrine cells that are also immunopositive for pancreostatin. Neither colocalization of ghrelin and enterochromaffin-like cells nor colocalization of ghrelin and D cells was found in the pancreas. Therefore, it was concluded that the ghrelin-positive cells must belong to the third subpopulation, the A cells (124). We have recently shown that ghrelin is produced by a fraction of endocrine pancreatic cells, namely the insulin-producing H cells, as confirmed by double immunofluorescence studies (169). Ghrelin mRNA and ghrelin peptide have also been detected in rat and human placenta in which they are expressed predominantly in cytotrophoblast cells and very sporadically in syncytiotrophoblast cells. A pregnancy-related time course, represented by an early rise of ghrelin expression in the third week and decreasing in the latest stages of gestation, but still detectable at term, was found in rats. In human placenta, ghrelin is mainly expressed in the first half of pregnancy and is not detectable at term (5). Involvement of ghrelin in fetal-maternal interaction via autocrine, paracrine, or endocrine mechanisms is discussed (5). In addition to the presence of GHS-R in pituitary cells, ghrelin mRNA expression and ghrelin immunopositive cells were detected in normal pituitary cells as well as in pituitary tumors (6, 22, 163). This suggests a possible autocrine or paracrine role for hypophyseal ghrelin, although only about 5% of the detected ghrelin peptide derived from the pituitary has been found to be octanoylated (6, 163); Ghrelin synthesis has been shown by the use of real-time PCR and direct sequencing of the PCR product in corticotroph, thyrotroph, lactotroph, and somatotroph cells of the pituitary (6, 163), whereas the highest levels of ghrelin expression were found in nonfunctioning adenomas, moderate ghrelin levels were found in GH-producing adenomas and gonadotropin-producing adenomas, and the lowest level was found in prolactinomas (6, 163). The same group detected small amounts of ghrelin in the adrenal glands, esophagus, adipocytes, gall bladder, muscle, myocardium, ovary, prostate, skin, spleen, thyroid blood vessels, and liver using real-time PCR (22). By combinatory use of reverse-phase HPLC and RIA of purified aliquots, production of ghrelin in mouse kidney was shown in greater abundance than in mouse plasma. In addition, preproghrelin production was shown in rat mesangial cells and mouse podocytes, indicating the production of ghrelin in kidney, glomerulus, and renal cells and suggesting possible paracrine roles for ghrelin in the kidney (4). In several regions of the brain, ghrelin was detected by means of immunohistochemistry. However, the location of the few ghrelin-positive neurons that were identified depends on the recognized epitope of the ghrelin antiserum used (51, 207). Very recently, ghrelin expression has been demonstrated in a previously uncharacterized group of neurons adjacent to the third ventricle. These neurons send efferents onto NPY, AGRP, proopiomelanocortin (POMC), and CRH neurons, suggesting that local ghrelin would represent a novel regulatory circuit controlling energy homeostasis (Fig. 3) (123). However, ghrelin found in the hypothalamus still has to be considered as possibly derived from the periphery, and the participation of hypothalamic ghrelin in neuropeptidergic energy balance control mechanisms remains questionable (51). Human ghrelin as well as GHS-R mRNA expression was shown by real time-PCR and confirmed by DNA-sequencing in human T-lymphocytes, B-lymphocytes, and neutrophils from venous blood of healthy volunteers. Large interindividual differences in ghrelin mRNA expression levels were described; however, cell type and maturity of the cells did not seem to have an influence on ghrelin production in immune cells (9, 208). Interestingly, it has recently been shown that small-molecule GHS have a considerable immune-enhancing effect (9, 208).

In summary, ghrelin is expressed primarily by the stomach and secondarily by lower parts of the gastrointestinal tract. Ghrelin expression levels in other organs are relatively low in comparison, and although its physiological significance as a paracrine factor in these tissues is the subject of ongoing studies, an endocrine role for extragastrointestinal ghrelin appears to be unlikely. Published studies on the regulation of ghrelin expression have therefore primarily focused on gastric ghrelin. Additional caution, however, has to be used by extrapolating from studies on ghrelin expression or secretion in rodents to the physiological regulation of ghrelin in humans.

Only a few determinants of circulating ghrelin concentration have been identified to date. Spontaneous ghrelin secretion is pulsatile in rats (44), and 24-h ghrelin variation is reported in humans by some (202, 209), but not by others (210). It is unclear whether aging is a determinant of serum ghrelin concentrations. Ghrelin secretion is reported to be sexually dimorphic in humans, with women in the late follicular stage having higher levels than men (210). Among determinants of ghrelin secretion, the most important appear to be insulin (211–217), glucose (33, 218–222), and SS (210, 223–227). Possibly, GH (224, 228–232), leptin (204, 233–237), melatonin (238), thyroid hormones (239), glucagon (240), and the parasympathetic nervous system (32, 241) also play a role in ghrelin metabolism. In mice, rats, cows, and humans, ghrelin mRNA expression levels or circulating ghrelin levels are increased by food deprivation and appear to be decreased postprandially (33, 137, 202, 209, 242–246). This phenomenon, which has been confirmed by several study groups in the recent past, further supports the emerging concept of ghrelin as an endogenous regulator of energy homeostasis. In addition to fasting, ghrelin expression can be stimulated in rats by insulin-induced hypoglycemia, leptin administration, and central leptin gene therapy (233, 243). Ingestion of sugar suppresses ghrelin secretion in rats (33). These observations indicate a direct inhibitory effect of glucose/caloric

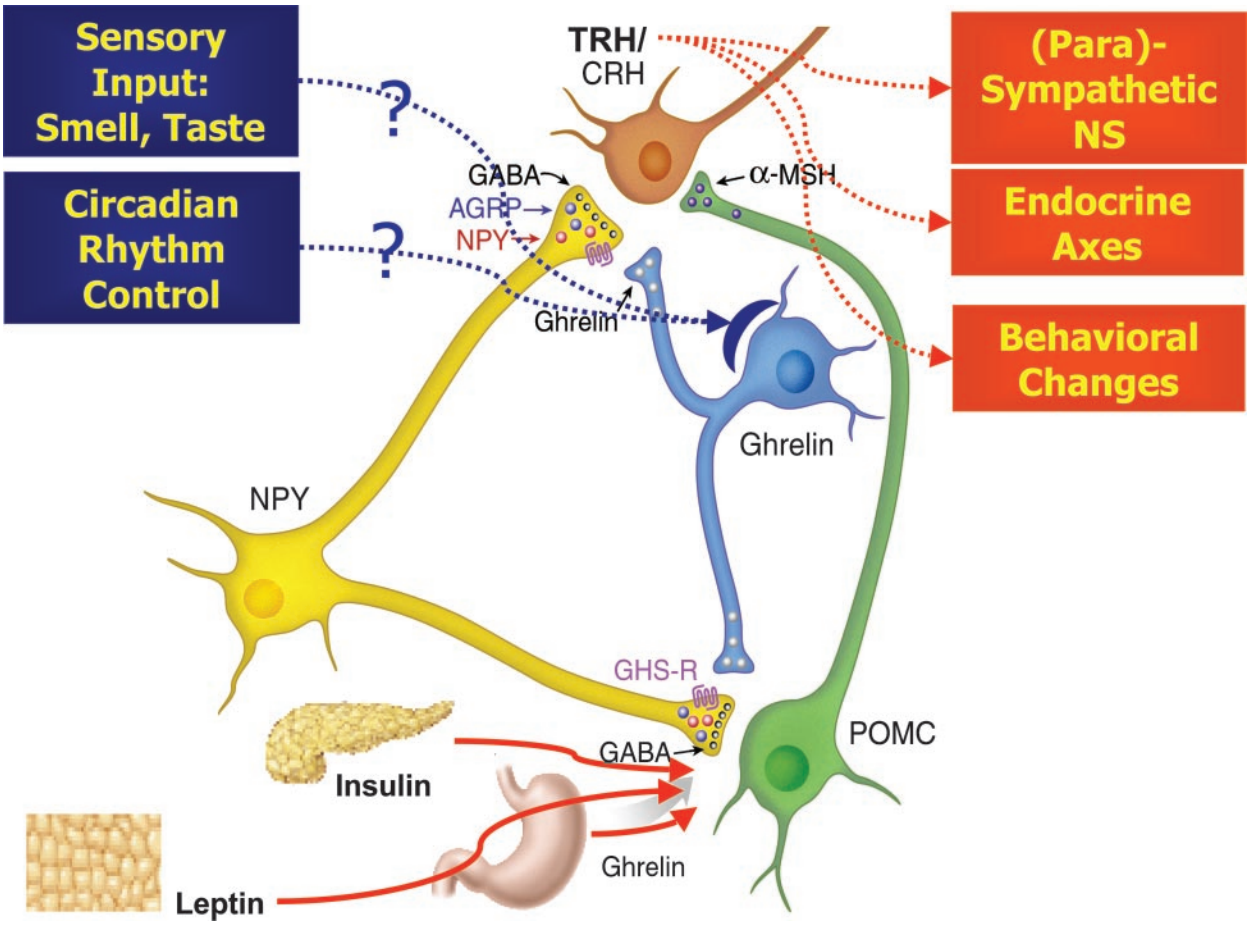

FIG. 3. Within a complex neuroendocrine network, afferent signals from the periphery are continuously indicating acute and chronic changes of energy balance, whereas integrative regulatory circuits in the CNS are modulating efferent pathways to adjust orexigenic drive, energy expenditure, and nutrient metabolism accordingly. Ghrelin is thought to be significantly involved in this neuroendocrine network regulating energy balance in at least two ways: 1) as a peripheral hormone from the stomach that, along with other signals such as insulin or leptin, informs the central energy balance control when energy stores diminish, to increase orexigenic drive and decrease energy expenditure; and 2) as a hypothalamic neuropeptide expressed in previously unidentified population of neurons adjacent to the third ventricle between the ventromedial hypothalamus, the dorsal hypothalamus, the paraventricular nucleus, and the arcuate nucleus. Efferents of ghrelin-expressing neurons project to key circuits of central energy balance regulation and may balance the activity of orexigenic NPY/AGRP with anorectic POMC neurons to modulate a resulting efferent message, which is believed to be mediated in part by TRH and CRH. *Dotted lines* indicate indirect effects or actions, whereas *question marks* indicate unproven actions (51, 233, 351, 352, 353).

intake on ghrelin-containing X/A-like cells in the oxyntic mucosa of the rat stomach rather than an exclusively insulin-mediated effect. That insulin is an independent determinant of the circulating ghrelin concentration has recently been shown by several study groups using hyperinsulinemic euglycemic clamps in humans (211, 212). These findings add further evidence connecting ghrelin to mechanisms governing energy balance and the regulation of glucose homeostasis.

Further insight into the apparently complex mechanisms regulating ghrelin secretion is based on studies showing an increase of circulating ghrelin levels in rats after surgical interventions such as vagotomy and hypophysectomy (32, 228). Human GHD, however, is not associated with increased plasma ghrelin levels (247). On the other hand, administration of synthetic GH to rats decreases circulating ghrelin levels, and therapeutic interventions causing normalization of GH levels in patients with acromegaly increase ghrelin levels (228–230). These partial, somewhat contradictory, observations could be due to species-specific differences between rodents and humans, or they could indicate that an acute, but not chronic, change of GH levels modulates ghrelin concentration. An increase in circulating ghrelin levels in rats with age, up to 90 d (248), has not been confirmed as yet for human populations. Early studies seem to indicate, however, that human ghrelin secretion decreases with age during childhood (249). A pathophysiological factor that might increase circulating ghrelin levels is the production of ghrelin by certain endocrine tumors of the stomach and the intestine such as carcinoids (126). A recent, very intriguing series of clinical studies by Cummings *et al.* (202, 209) indicates that each daily meal is followed by decreases of circulating ghrelin levels, most likely reflecting acutely reduced ghrelin secretion from the gastrointestinal tract. The authors speculate in addition that an observed premeal rise of circulating human ghrelin levels might reveal a role for ghrelin in meal initiation. This theory fits well with the observation that ghrelin administration in healthy volunteers causes hun-

ger (15, 16, 35). Ghrelin might also reflect the acute state of energy balance, signaling to the central nervous system (CNS) in times of food deprivation that increased energy intake and an energy-preserving metabolic state are desirable (33, 51). In addition, one biological purpose of these multiple roles of ghrelin might be to ensure the provision of calories that GH requires for growth and repair.

In summary, ghrelin expression and ghrelin secretion are mainly influenced by changes in energy balance and glucose homeostasis, followed by alterations of endocrine axes such as increasing GH concentrations. Based on the currently available data, ghrelin therefore seems to be part of a molecular regulatory interface between the energy homeostasis, glucose metabolism, and physiological processes regulated by the classical endocrine axes such as growth and reproduction.

V. Physiological and Pathophysiological Actions of Ghrelin

A. Hypothalamic-pituitary actions

1. GH-releasing activity. Ghrelin and synthetic GHS possess strong and dose-related GH-releasing activity that is more marked in humans than in animals (1, 14–16, 18, 20, 250–252). Natural and synthetic GHS stimulate GH release from somatotroph cells *in vitro* (1, 62, 65, 253–265), probably by depolarizing the somatotroph membrane and by increasing the amount of GH secreted per cell (258). A stimulatory effect of GHS on GH synthesis has also been reported by some authors (260). *In vitro*, the GH-releasing activity of GHS is lower than that of GHRH (62, 65, 254, 266). Under this condition, an additive or a true synergistic effect of GHS on GHRH-stimulated GH has been reported (65, 254–256, 262, 263). At the pituitary level, the stimulatory effect of GHS on GH secretion from somatotroph cells is abolished by specific GHS antagonists but not by GHRH antagonists (65, 255, 258). SS inhibits the stimulatory effect of GHS on GH secretion from pituitary (253, 254, 266, 267). However, there is evidence suggesting that GHS could act by antagonizing the inhibitory activity of SS on GH release by counteracting its hyperpolarizing effect on somatotroph cell membranes (258).

The GH-releasing activity of GHS is clearly greater in hypothalamic-pituitary preparations than in pituitary preparations (268), in agreement with evidence that their stimulatory effect on GH secretion is greater *in vivo* than *in vitro* (65, 269). Indeed, *in vivo*, GHS show synergistic effects on GHRH-stimulated GH release (65, 270) and prevent the normal cyclic refractoriness to GHRH (269). To confirm that the most important action of ghrelin and synthetic GHS to release GH takes place at the hypothalamic level, the GH-releasing effect of GHS is markedly reduced, although not abolished, in animals with lesions of the pituitary stalk (271–273).

At the hypothalamic level, ghrelin and GHS act via mediation of GHRH-secreting neurons as indicated by evidence that passive immunization against GHRH, as well as pretreatment with GHRH antagonists, reduces their stimulatory effect on GH secretion (65, 269, 274–276). An increased release of GHRH in portal blood of the pituitary after GHS administration has also been shown in sheep (277). In terms of GH release, GHS are active in dwarf mice (278) but not in the lit/lit mouse, which has no pituitary GHRH receptors (279). However, in both GHD rats and lit/lit mice, systemic administration of GHS activates a subpopulation of hypothalamic arcuate neurons where the highest density of GHRH-secreting neurons is present. Furthermore, because the lit/lit mouse pituitary does not release GH after GHS administration, the finding that the central actions of GHS remain intact in these animals suggests the possible existence of two subpopulations of putative GHS-Rs (280, 281).

At the hypothalamic level, ghrelin and GHS do not inhibit SS secretion *in vitro* in rats; however, some inhibition of hypothalamic SS secretion after exposure to GHS was observed *in vivo* in pigs (137, 227, 282–284). Interestingly, GHS likely act as functional SS antagonists at either the hypothalamic or the pituitary level (71, 258). *In vitro* and *in vivo*, GHS and GHRH induce homologous but not heterologous desensitization (61, 65, 78, 254, 255, 262, 263, 266, 269). Prolonged administration of GHS in animals increases IGF-I levels (61, 87, 93, 267, 285, 286), indicating that they are able to enhance the activity of the GH/IGF-I axis.

As anticipated, ghrelin and synthetic GHS show their most potent GH-releasing activity in humans (1, 14–16, 18, 20, 250–252) and in animals *in vivo* because GHS and GHRH are synergistic, indicating that they act, at least partially, via different mechanisms (14, 20, 71, 120). Nevertheless, GHS require GHRH activity to fully express their GH-releasing effect (14, 20, 71, 120). In humans, the GH response to GHS is strongly inhibited, although not abolished, by GHRH receptor antagonists as well as by hypothalamopituitary disconnection (272, 287–289). This is in agreement with the assumption that the most important action of GHS takes place at the hypothalamic level (14, 20, 61, 63, 65, 120). Moreover, patients with a GHRH receptor deficiency show no increase in GH secretion in response to GHS stimulation (290–292) but maintain their ability to increase PRL as well as ACTH and cortisol secretion after GHS stimulation (290–292).

There is evidence, both in humans and in animals, that ghrelin and synthetic GHS can also act as functional SS antagonists at both the pituitary and hypothalamic levels (20, 71, 191, 218, 227, 258, 293). In fact, in humans the GH response to ghrelin and GHS is not enhanced by inhibition of SS release (induced by indirect cholinergic agonists or arginine), whereas it is partially refractory to the inhibitory effect of substances acting via stimulation of hypothalamic SS secretion (such as acetylcholine receptor antagonists, β-adrenoreceptor agonists, glucose) (20, 191). Indeed, ghrelin and GHS are even partially refractory to the inhibition of substances that act on the pituitary somatotroph cells, such as free fatty acids and even to exogenous SS (20, 191, 293, 294). GHS are also partially refractory to the negative feedback of GH itself and to the negative feedback of IGF-I action (193, 295, 296).

In humans, as in animals, there is evidence that GHS and GHRH induce homologous, but not heterologous, desensitization (64, 218, 261, 297–302). Homologous desensitization to the activity of GHS has been shown during GHRP infusion (297, 299, 300), but not after intermittent oral or intranasal daily administration of the peptide for up to 15 d (303, 304).

On the other hand, prolonged administration of GHS by the parenteral, intranasal, or oral route enhances spontaneous GH pulsatility over 24 h and increases IGF-I levels in normal young adults, as well as in short children and elderly subjects (11, 85, 86, 299, 300, 304–306).

The GH-releasing effect of GHS undergoes marked age-related variations, increasing at puberty. It plateaus in adulthood and decreases during further aging (20). The mechanisms underlying these variations differ by age. The enhanced GH-releasing effect of GHS at puberty, for instance, is caused by the positive influence of increased serum estrogen levels, which increase GHS-R expression (110, 196, 307–311). However, estrogen insufficiency does not fully explain the reduced GH response to GHS in postmenopausal women (20, 196, 312–314). In agreement with the reduction in hypothalamic GHS-Rs in human aging, the GH response to hexarelin in elderly subjects is further increased, but not restored, by supramaximal doses (20, 24, 307). The most important mechanism accounting for reduced GH-releasing activity of GHS in aging is probably represented by age-related variations in neural control of somatotroph function, including GHRH hypoactivity and somatostatinergic hyperactivity (20, 314). On the other hand, it has also been hypothesized, but not proven, that the age-related decline in GH secretion might reflect a decrease in activity of the endogenous GHS system (*i.e.*, ghrelin release and/or receptor expression) (24, 70, 314, 315). As with GHS, the GH-releasing effect of ghrelin is independent of gender but undergoes age-related decrease. Again, the effect of ghrelin on lactotroph and corticotroph secretion is age and gender independent (316).

Despite the strong GH-releasing effects of ghrelin and GHS, whether GH release is the most important physiological action of ghrelin has been recently questioned. In fact, GHRH antagonist strongly inhibits 24-h GH secretion, whereas it does not affect circulating ghrelin levels (210). Moreover, ghrelin does not mediate the GH response to provocative stimuli such as insulin-induced hypoglycemia (213, 214), as well as GH rebound after withdrawal of SS infusion (223). These observations are in agreement with evidence from animal studies showing that ghrelin secretion is pulsatile and is associated much more with food intake than with GH pulses (44).

Theoretically, ghrelin or GHS could have diagnostic and therapeutic implications based on the strong and reproducible GH-releasing effects of orally active GHS.

Particularly when combined with GHRH, ghrelin and GHS can be used for one of the most potent and reliable stimulation tests to evaluate the capacity of the pituitary to release GH for the diagnosis of GHD (252, 317–320). Provided that appropriate cut-off limits are established, these tests using GHS for the diagnosis of GHD are as sensitive and specific as an insulin tolerance test, the gold standard test for the diagnosis of GHD (252, 318, 319). Long-acting and orally active ghrelin analogs might represent an anabolic treatment in frail elderly subjects. This potential treatment modality is based on the rationale that age-related reduction in the activity of the GH/IGF-I axis probably accounts for changes in body composition, structure functions, and metabolism in normal elderly subjects that are remarkably similar to those in GHD adults (321, 322). Also, the potential pituitary GH release in aged subjects is still remarkably intact, given the fact that the appropriate stimuli are used (322). Finally, GH-releasing substances would represent a more physiological approach to increase endogenous GH pulsatility than a single daily dose of recombinant human GH (321, 322).

At present, there is no definite evidence that shows the therapeutic efficacy of ghrelin analogs as anabolic agents acting via rejuvenation of the GH/IGF-I axis in elderly subjects, although some benefits in osteoporosis have been reported (99, 102).

2. PRL- and ACTH-releasing activities. Activity of both ghrelin and synthetic GHS is not fully specific for GH, because it also includes stimulatory effects on both the lactotroph and corticotroph system (16, 18, 20, 63, 250, 323, 324). However, some synthetic GHS that exclusively stimulate GH secretion have been reported (106). The stimulatory effect of ghrelin and its analogs on PRL secretion in humans is far less age and gender dependent than the effect on GH secretion (316).

The stimulatory effect of GHS on the activity of the hypothalamus-pituitary-adrenal axis in humans is remarkable and similar to that of the administration of naloxon, vasopressin, and even CRH. Interestingly, the effect of ghrelin on ACTH secretion is even more pronounced than that elicited by synthetic GHS (16, 18, 69, 325–330). However, this ACTH release after GHS administration appears to be an acute neuroendocrine effect that attenuates during prolonged treatment (14, 20, 70, 85, 86, 285, 331).

The GHS-induced ACTH release is independent of gender but shows peculiar age-related variations (331). It increases at puberty, then shows a reduction in adulthood and, again, a trend toward an increase in aging when the GH response to these molecules is clearly reduced (191, 316, 325, 331).

Under physiological conditions, the ACTH-releasing activity of GHS is entirely mediated via the CNS (20, 272, 331, 332). These mechanisms via the CNS not only include CRH- and/or vasopressin-mediated actions (20, 137, 325, 327, 328, 330, 331, 333) but also via NPY and/or γ-aminobutyric acid (GABA) (160, 283, 334). The ACTH response to natural and synthetic GHS is generally sensitive to the negative cortisol feedback mechanism (20, 105, 331, 334). However, the stimulatory effect of ghrelin and GHS on corticotroph secretion is exaggerated and higher than that of human CRH in patients with pituitary ACTH-dependent Cushing's disease, probably reflecting a direct action of ghrelin and GHS on the pituitary ACTH-secreting tumor cells (20, 121, 331, 335–337). Interestingly, the administration of CRH to humans does not induce any significant increase in ghrelin secretion (230). In agreement with the presence of ghrelin and GHS-R expression in ectopic ACTH-secreting tumors, exaggerated ACTH and cortisol response to GHS has also been observed in patients with ectopic ACTH-dependent Cushing's syndrome (121, 163, 166, 331). These observations, however, reduce the potential use of GHS in testing ACTH secretion to distinguish patients with pituitary from ectopic ACTH-dependent hypercortisolism.

B. Central actions of ghrelin and GHS

1. Effects on food intake. Years before ghrelin was discovered, sporadic and greatly neglected reports on observations in rodents indicated that some GHS might possess orexigenic activity (338–341). Rumors about GHS-induced ravenous hunger attacks in children with idiopathic short stature occurring within the framework of clinical studies on GHS have never been officially confirmed. A research group led by S. Dickson had, however, gathered a substantial amount of very intriguing data during the last decade showing GHS-induced neuronal activity in hypothalamic areas that are currently considered the central processing unit controlling energy balance (160, 199, 279, 280, 342–345). A dense expression of the G protein-coupled receptor GHS-R 1a has been shown on those neurons, and it is bound and activated by ghrelin as well as by other GHS and GHRP (13, 113, 114, 158). Still, it was a surprise to many when ghrelin, the endogenous ligand of the GHS-R (1, 143), emerged as one of the most powerful orexigenic and adipogenic agents known in mammalian physiology (33, 34, 52, 70). At first, it was puzzling to link adipogenic effects to a hormone that had originally been discovered as a potent secretagogue of a lipolytic hormone, GH (346, 347). However, a rapidly growing body of data reflecting a previously unidentified interface between energy balance regulation, glucose homeostasis, and hypothalamic neuropeptides started to make sense as an evolving mosaic drawn together by ghrelin. Similar to the discovery of the satiety effects of leptin that indicated adipocytes as endocrine organs, the observation of ghrelin's involvement in energy balance regulation is pointing to an additional endocrine role for the stomach as well (1, 124). Very recently, ghrelin expression was found in a previously uncharacterized group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei. These neurons send efferents onto key hypothalamic circuits, including those producing NPY, AGRP, POMC, and CRH. Within the hypothalamus, ghrelin mostly stimulated the activity of arcuate NPY neurons in the paraventricular nucleus, so the release of ghrelin may stimulate the release of orexigenic peptides and neurotransmitters, thus representing a novel regulatory circuit controlling energy homeostasis (Fig. 3) (123, 348–352).

Ghrelin administration in rodents causes weight gain (33, 39, 40, 353). This effect would not be as astonishing if it was merely reflected by longitudinal growth or at least by an increase in lean mass, effects that one would expect to occur after stimulation of GH secretion (354). However, a still growing amount of data generated in rodents clearly showed that ghrelin-induced weight gain is based on accretion of fat mass without changes in longitudinal skeletal growth and with a decrease, rather than an increase in lean (muscle) mass (33). These findings have not only been confirmed by several groups but have also been repeated using synthetic ghrelin receptor (GHS-R) agonists NNC 26-0161 (ipamorelin), GHRP-2, and GHRP-6 (353, 355). Changes in body weight induced by ghrelin administration become significant in rodents after no more than 48 h and are self-evident at the end of 2 wk (33, 353). Changes in fat mass induced by GHS have been quantified using dual energy x-ray absorptiometry measurements specifically adapted for analysis of rodent body composition and have also been confirmed using chemical carcass analysis (33, 353, 355), or by measuring the weight of omental and retroperitoneal fat pads (355). Currently, it seems likely that the effects that are causing a positive energy balance are mediated via leptin-responsive neurons in specific regions of the hypothalamus (31, 39, 51, 159, 199, 353, 356, 357). However, the possibility of direct effects of ghrelin on adipose tissue [where GHS-R mRNA expression has been shown by PCR (1)], as well as effects on the hypothalamus-pituitary-adrenal axis (20, 34), *i.e.*, a ghrelin-induced Cushing's syndrome, still have to be ruled out as possible phenomena contributing to ghrelin-induced adiposity.

To find the physiological mechanism behind all these observations on ghrelin and energy homoeostasis, the rapidly evolving field of research focusing on body weight and appetite regulation (233, 356, 358–360) has to be integrated with existing knowledge regarding GHS and their actions (70, 73, 314). Energy balance is achieved when energy intake is equal to energy expenditure (356). A positive energy balance, leading to weight gain, occurs when calories ingested, digested, and reabsorbed exceed calories expended (356). Like leptin, but in an opposite manner, ghrelin administered to rodents influences both energy intake and metabolism (51).

The earliest published data on the orexigenic effects of GHS are from Locke *et al.* (338), showing an increase in food intake after intracerebroventricular administration in rats without affecting plasma GH response. Similar effects have been shown by several other groups (339–341, 355). These effects were described as most likely being independent of GH and could not be prevented by blockade of the GHRH pathway (340).

Once ghrelin was found, we observed that it stimulated food intake in rodents (33). This effect is dose-dependent and occurs more powerfully after central than after peripheral administration (33), suggesting a central mechanism of action.

The increase in food intake after ghrelin injection in rodents occurs rapidly [less than 60 min (34)], which causes this effect to be easily missed by traditional methods of daily food intake measurements (measurement of food weight every 24 h). The orexigenic action of ghrelin (when administered centrally) is comparable to that of the brain-derived NPY and is more potent than that of any other orexant (34). Although peripherally injected GHS or ghrelin does have less impressive (353), predominantly acute, and maybe solely temporary orexigenic effects, ghrelin continuously administered into the third ventricle causes potent and constant stimulation of appetite in rats (33). However, further studies (*i.e.*, involving mice with tissue-specific disruption of the ghrelin gene) will have to prove the existence of an endogenous ghrelin tone that supports the putative relevance of ghrelin for physiological appetite regulation and metabolic control. Some experiments involving central administration of ghrelin antiserum or GHS-R antagonists (39) already provide some framework for this concept.

In a series of elaborate studies, Shuto *et al.* (361) recently showed that expression of antisense GHS-R mRNA under the control of the promoter for tyrosine hydroxylase in transgenic rats selectively attenuates GHS-R protein expression in

the arcuate nucleus and consecutively decreases GH secretion, food intake, and body fat mass, because these antisense GHS-R mRNA-expressing rats had lower body weight and less adipose tissue than the control rats. Moreover, daily food intake was reduced, and the stimulatory effect of GHS treatment on feeding was abolished. These results, however, should be interpreted with caution because of the potential for limited specificity when an antisense approach is used to generate transgenic animals. Anyway, GHS that have been shown to have orexigenic activity so far include GHRP-2, ipamorelin, GHRP-6, hexarelin, and several of its analogs (34, 340, 341, 353, 355).

Unlike other comparably potent orexigenic agents (NPY, AGRP, melanin-concentrating hormone) that are solely active when injected into the brain (233, 362, 363), peripherally administered synthetic ghrelin and ghrelin receptor analogs still exhibit orexigenic and adipogenic effects (70). This does not necessarily mean that an acylated peptide such as ghrelin is capable of being transported across the blood-brain barrier. Even if early studies indicate that miniscule amounts of ghrelin are transported across the blood-brain barrier in the blood-to-brain direction, (198, 364, 365), ghrelin might still have some of the hypothalamic actions because those hypothalamic areas that are crucial for the regulation of energy homeostasis, such as the ventromedial part of the arcuate nucleus, may not be completely protected by the blood-brain barrier (Fig. 4) (366). Some of these areas (*i.e.*, the ventromedial arcuate nucleus) may therefore be accessible by molecules in the circulation (51). These hypothalamic nuclei intriguingly contain neurons that express the GHS-R (158) and might therefore be essential for the mediation of effects triggered by gastric or peripherally injected synthetic ghrelin (51, 70). The validity of this concept has been confirmed by Dickson and colleagues (161), who demonstrated that peripherally injected ghrelin induces increased expression of the early gene products c-Fos and EGR-1 in NPY-, AGRP-, and GHS-R-coexpressing neurons in the arcuate nucleus.

The network of neurons, neuropeptides, and receptors controlling energy balance is an extremely complex, multicentered system (233). Based on early surgical and chemical deletion studies in rodents, the hypothalamus has long been recognized as a crucial interface between afferent peripheral signals, CNS wiring, and efferent neuroendocrine axes regulating energy balance in concert (356). Several recent reviews give excellent overviews on the principles in this fascinating and rapidly advancing field (233, 356, 358, 359).

According to current knowledge, it seems that two major hypothalamic pathways are the predominant mediators of ghrelin's influence on energy balance (39, 159, 353). One involves the NPY neurons (37, 362), and the other involves the melanocortin receptors and their agonistic and antagonistic ligands, the anorexigenic POMC-derived αMSH and the orexigenic AGRP, which is expressed in NPY neurons (367). Ghrelin increases AGRP and NPY after acute and chronic administration, and hypothalamic AGRP-mRNA expression levels are found to be up-regulated after chronic activation of the GHS-R for several weeks (31, 37, 39, 353). Complete absence of NPY in NPY-gene-disrupted mice does not influence ghrelin action. This virtual contradiction could also be explained by adaptive processes during the early development of the NPY-gene-disrupted mice (368). Some studies show a prevention of the orexigenic effects when coadministering a NPY receptor antagonist with ghrelin (353); however, it has to be considered that NPY antagonist might also be acting through a more dominant pathway than the one controlled by ghrelin. We assume that the two pathways described above comediate the effects of ghrelin on energy balance, and furthermore speculate that NPY might be more important for acute effects, whereas AGRP might be

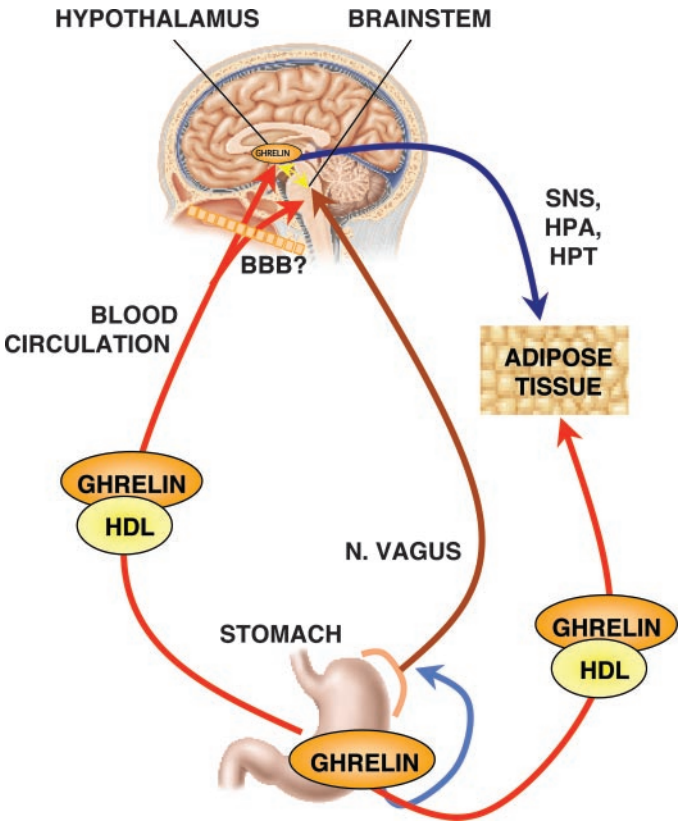

FIG. 4. Pathways by which ghrelin may influence chronic energy balance. Ghrelin produced by the stomach or the gut can be transported by the bloodstream to specific neuronal circuits situated in hypothalamus or the brainstem that are regulating food intake as well as energy expenditure. It still remains uncertain whether or not ghrelin has to cross the blood-brain barrier (BBB) to influence these central structures. During transport via circulating blood, serum HDLs and presumably other proteins such as albumin bind ghrelin. Ghrelin however may also signal the brain by activating the afferent vagal nervous system as either an endocrine or a paracrine signal directly at the stomach level. Ghrelin-responsive GHS-Rs are expressed at gastric vagal nerves, and vagotomy prevents some of ghrelin's effects on energy balance. Incoming information represented or triggered by ghrelin is, however, believed to be constantly sensed and analyzed in hypothalamus and the brainstem, independent from its origin or afferent pathway used. Based on constant integration of this and other afferent information about the status of acute and chronic changes in energy balance, an efferent response seems to involve several pathways to balance energy stores and adipose tissue mass. These mainly include the sympathetic nervous system (SNS), the hypothalamic-pituitary adrenal (HPA) axis, and the hypothalamic-pituitary thyroid (HPT) axis. In addition, ghrelin is thought to be produced in brain centers of energy balance control, and, although present there in very small amounts, brain-derived ghrelin might play an additional role in the regulation of energy homoeostasis (51, 123, 152, 198, 372).

involved in both chronic and acute ghrelin action in the hypothalamus (51).

Other agents possibly mediating ghrelin signals in the hypothalamus are POMC, cocaine and amphetamine-regulated transcript, melanin-concentrating hormone, orexin (hypocretin) a/b, ciliary neurotrophic growth factor, GABA, and galanin (51, 356, 358).

Apart from an increase of food intake, other mechanisms can contribute to an increase in fat mass, such as a decrease in energy expenditure or reduced cellular fat oxidation (356). No significant changes of 24-h energy expenditure have been observed in rodents after ghrelin administration. Another effect that was detected by indirect calorimetry is an impressive increase of the respiratory quotient after ghrelin administration in rodents, independent of an increase in food intake (33). This phenomenon is interpreted as a shift from fat utilization to carbohydrate oxidation and has been referred to as nutrition partitioning (369).

Furthermore, there is some published evidence that ghrelin action might be mediated in part not only by efferent but also by afferent activity of the vagal nerve (137, 370). These data were generated by electrophysiological studies, in which iv administered ghrelin has been shown to decrease the afferent activity of the gastric vagal nerve at low doses (137). The described effects are in opposition to those of gastrointestinal satiety peptides such as cholecystokinin (371) and may add additional pathways to the growing number of signaling routes with which ghrelin is connected (Fig. 1, A and B) (52). More recently, it has been reported that blockade of the gastric vagal afferent abolished ghrelin-induced feeding, GH secretion, and activation of NPY-producing and GHRH-producing neurons in rats. This study suggested that the gastric vagal afferent is the major pathway conveying ghrelin's signals for starvation and GH secretion to the brain (372). However, it has to be emphasized that muscarinic receptor blockade by pirenzepine does not affect at all the endocrine activities of ghrelin in humans (373).

In summary, administration of ghrelin and of at least some of its receptor agonists generates a positive energy balance and increases adiposity in rodents via multiple mechanisms, including increased food intake and reduced fat oxidation, possibly along with decreased locomotor activity and adipocyte-specific effects. In addition, expression of GHS-R in rat adipocytes increases with age and during adipogenesis. Ghrelin *in vitro* stimulates the differentiation of preadipocytes and antagonizes lipolysis. Ghrelin may therefore play an important role in the process of adipogenesis in rats (374).

Possibly the most pressing question concerns the transferability and validity of the above-described findings in rodents to humans. Several recent clinical studies on the effects of ghrelin on GH secretion in humans have reported hunger sensations as the only noticeable side effect in up to 80% of the treated individuals (15, 35). Prospective clinical studies focusing on all aspects of energy balance using contemporary methods for the analysis of body composition, energy expenditure, metabolic and endocrine changes can help to clarify these issues. A first clinical study investigating these open questions was conducted by Wren *et al.* (40) who showed that iv administration of physiologically occurring concentrations of ghrelin effectively triggers appetite and increases food intake in humans.

2. Effects on sleep. Alterations in the sleep pattern are a hallmark of functional correlates reflecting age-related changes in neurotransmitters and neuropeptides (375). A potential influence of GH and IGF-I on sleep pattern has also been suggested based on studies in GHD subjects, and it had been hypothesized that sleep pattern in aged subjects could reflect an age-related decrease in the activity of the GH/IGF-I axis (375). On the other hand, some studies reported that the acute administration of synthetic, peptidyl GHS can modify sleep pattern in normal subjects (92, 376, 377). Moreover, it has also been reported that prolonged treatment with oral MK-0677 (25 mg once daily) in elderly subjects increases the length of rapid eye movement sleep phases, meanwhile decreasing rapid eye movement latency (378). These findings from early studies with synthetic GHS agree with ongoing studies addressing the influence of ghrelin on sleep behavior (44, 379). In rats, there is evidence as well that ghrelin also affects sleep-wake patterns (44). Furthermore, ghrelin itself has been reported recently to be a sleep-promoting factor in humans (379).

3. Effects on behavior. Besides regulating eating behavior, it has recently been shown that ghrelin affects anxiety-like behavior in mice, via mechanisms involving the hypothalamic-pituitary-adrenal axis. Both intracerebroventricular and ip administration of ghrelin potently induce anxiogenic activities. In addition, administration of a CRH receptor antagonist significantly inhibits ghrelin-induced anxiogenic effects. Peripherally administered ghrelin significantly increases CRH mRNA expression in the hypothalamus. These findings suggest that ghrelin may have a role in mediating neuroendocrine and behavioral responses to stressors and that the stomach could play an endocrine role, not only in the stimulation of appetite, but also in the induction of anxiety (45).

C. Peripheral activities of synthetic and natural GHS

In agreement with the existence of specific ghrelin receptors in peripheral tissues (see *Section III. A*), peripheral endocrine and nonendocrine activities of these substances have been recently demonstrated. Apart from a potent GH-releasing effect, ghrelin and synthetic GHS control gastric motility and acid secretion, influence endocrine pancreatic function, alter glucose metabolism and cardiovascular functions, regulate adipocyte modulation of adipokines, and have antiproliferative effects in neoplastic thyroid, mammary, and lung cell lines (Fig. 2).

1. Gastroenteropancreatic actions. Gastrectomy in rats reduces circulating ghrelin concentration by approximately 80%, in agreement with the assumption that the stomach is the main source of the endogenous GHS-R ligand (3, 124, 230). As discussed elsewhere in this review, small quantities of ghrelin are also expressed in other enteric tracts, as well as in the pancreas (1, 22, 124–126, 128, 135, 150, 169, 200, 206, 243, 245, 380–386).

It is not surprising that ghrelin also acts at the gastroenteropancreatic level, where GHS-R 1a and 1b expression has

been demonstrated (3, 7, 22, 30, 126, 150, 157, 200, 243). Interestingly, there is also a close structural relationship between the precursors of motilin and ghrelin, but only a 36% homology exists between the mature peptides (137). Also, the gastrointestinal motilin receptor 1a and the GHS-R 1a show a high degree of structural homology (112, 118). The similarity between ghrelin and motilin actions has recently been emphasized (112, 127, 134–140, 382, 387) and has been discussed elsewhere in this review. Also, circulating levels of ghrelin and glucagon-like peptide (GLP)-1 seem to be inversely related during glucose ingestion (388, 389). The clinical implication of this has not been determined.

Ghrelin stimulates gastric acid secretion and motility in rats (32, 136, 390), and circulating ghrelin levels are correlated with gastric emptying time in humans (242). However, Sibilia *et al*. (391) reported a centrally mediated inhibitory role of ghrelin and synthetic GHS on acid secretion in rats. The stimulatory actions of ghrelin are mediated by the cholinergic system because they are abolished by muscarinic blockade (32). Interestingly, the acetylcholine-mediated stimulatory effect of ghrelin on gastric acid secretion takes place, at least partially, at the central level (36). More recently, it has been clarified that stomach-derived ghrelin's signals for starvation and GH secretion are relayed to the brain by means of the vagal nerve (372). However, in humans, cholinergic blockade by pirenzepine has been shown unable to modify the endocrine activities of ghrelin (373). Interestingly, the potent prokinetic activity of ghrelin/motilin-related peptide has been recently confirmed and extended to allow for reversal of a gastric postoperative ileus in rats (136, 392).

Ghrelin and GHS-R 1a mRNA are present in normal and neoplastic endocrine pancreas (22, 30, 126, 163, 206, 393, 394).

Regarding the exocrine pancreas, it has been shown that ghrelin is a potent inhibitor of pancreatic cholecystokinin-induced exocrine secretion in anesthetized rats *in vivo* and in pancreatic lobes *in vitro*. These actions of ghrelin are indirect and may be exerted at the level of intrapancreatic neurons (42).

Regarding the endocrine pancreatic function, in addition to inconstant data on the expression of the classical GHS 1a (22, 30, 128, 169, 206), ghrelin has been demonstrated to be expressed by pancreatic endocrine α-cells in rat and human tissue by some authors (206) and by pancreatic β-cells by one group (169). It was reported that ghrelin is expressed in a quite prominent endocrine cell population in human fetal pancreas, and ghrelin expression in the pancreas precedes by far that in the stomach. Pancreatic ghrelin cells remain at lower numbers in adult islets. According to Wierup *et al*. (395), ghrelin is not coexpressed with any known islet hormone, and the ghrelin cells may therefore constitute a new islet cell type. Studies in animals reported conflicting results regarding the influence of ghrelin on insulin secretion (41, 206, 381). In fact, ghrelin was able to stimulate insulin secretion from isolated rat pancreatic islets (206) and *in vivo* (381, 393). On the other hand, insulin secretion from isolated rat pancreas, perfused *in situ* after stimulation with glucose, arginine, and carbachol, was found to be blunted by exposure to ghrelin that also reduced the SS response to arginine (41). These findings suggest that ghrelin exerts a tonic inhibitory regulation on insulin secretion from pancreatic β-cells, contributing at least in rats to a restrained release during food deprivation. In agreement with this hypothesis, a clear negative association between ghrelin and insulin secretion has been found in humans as well as in animals by the majority of authors (44, 209, 211, 243, 245, 396), although not by all (397). Again in agreement with the assumption that ghrelin negatively modulates pancreatic β-cell secretion, at least transiently, it has been demonstrated recently that in humans, ghrelin induces a significant increase in human plasma glucose levels that is followed, surprisingly, by a reduction in insulin secretion (35, 316, 398). Coupled with the observation that acute as well as chronic treatment with GHS, particularly nonpeptidyl derivatives, induced hyperglycemia and insulin resistance in a considerable number of elderly subjects and obese patients (90, 97, 399), these observations suggest that ghrelin is a gastroenteropancreatic hormone, exerting a significant role in the regulation of insulin secretion and glucose metabolism. Ghrelin might integrate the hormonal and metabolic response to fasting that, at least in humans, is connoted by a clear-cut increase in GH secretion coupled with inhibition of insulin secretion and activation of mechanisms devoted to maintaining glucose levels (231, 399, 400). The negative association between ghrelin and GLP secretion adds further credibility to this hypothesis (395), whereas future studies will need to show whether GLP influences ghrelin secretion and vice versa. Regarding glucose levels, it has already been shown that ghrelin likely blocks the inhibitory effects of insulin on gluconeogenesis (401). Also, ghrelin secretion may be suppressed, at least in part, by an increased plasma glucose level as well as by insulin *per se*, as shown by hyperinsulinemic euglycemic clamp studies in healthy subjects (211, 214, 218). However, this observation also suggested that ghrelin could have direct stimulatory effects on glycogenolysis, and this action is likely mediated by a non-GHS-R 1a process, because it is not exerted by synthetic GHS (35). All data show that ghrelin secretion seems to be negatively associated with body mass while its levels are increased in anorexia nervosa and cachexia. Also, early morning, overnight fasting ghrelin concentrations seem to be decreased in obese subjects. This indicates the potential major impact of ghrelin on insulin secretion and glucose metabolism suggested by studies summarized above (211, 214, 218, 231, 399–401). Ghrelin may play an important role in the process of adipogenesis, at least in rats. In fact, ghrelin administration significantly increases the levels of peroxisome proliferator-activated receptor-γ2 mRNA in primary cultured rat differentiated adipocytes. In addition, isoproterenol-stimulated lipolysis is significantly reduced by simultaneous ghrelin treatment in a dose-dependent manner *in vitro*. Moreover, ghrelin stimulates the differentiation of preadipocytes and antagonizes lipolysis (374).

In the context of the relationship between ghrelin and obesity, it is noteworthy that the only exception to the negative association between ghrelin and body mass is represented by obese patients with Prader-Willi syndrome (PWS) that show peculiarly elevated circulating ghrelin levels (402, 403). In a study by Haqq *et al*. (404) in children with PWS, fasting ghrelin concentrations were not significantly differ-

ent compared with normal weight controls but were higher than in obese children.

2. Cardiovascular and hemodynamic effects. The presence of GHS-R 1a mRNA has been demonstrated in heart and aorta (22, 38), and specific binding sites for ghrelin have been recently identified in rat heart and human arteries, where the density of ghrelin receptors is up-regulated with atherosclerosis (405). Considerable specific binding of radiolabeled peptidyl GHS (such as [^{125}I]Tyr-Ala-hexarelin and [^{125}I]Tyr-benzoylphenylalanine-hexarelin) is easily detectable in rat myocardium (107, 167) and in different human cardiovascular tissues (ventricles, atria, aorta, coronaries, carotid, endocardium, and vena cava), in quantities often higher than those found in the pituitary gland (24, 26). This binding is inhibited by unlabeled Tyr-Ala-hexarelin, hexarelin, and other peptidyl GHS, but not by the nonpeptidyl GHS MK-0677 or even by ghrelin, as well as by classical cardioactive substances (26). Therefore, these binding sites are unlikely to be classical GHS-Rs because they do not bind ghrelin (see *Section III. A*).

In agreement with the presence of GHS-Rs (both ghrelin and nonghrelin receptors) in the cardiovascular system, there is already evidence that ghrelin and (or) GHS mediate GH-independent cardiovascular activities, both in animals and in humans.

Although administration of high pharmacological doses of peptidyl GHS is reported to induce clear but transient coronary vasoconstriction in the perfused rat heart (49, 167), in young rats with selective GHD induced by passive immunization against GHRH, hexarelin pretreatment is able to protect against myocardial ischemic damage induced by low-flow ischemia and reperfusion (406, 407). Such a protective activity was associated with a recovery of prostacyclin release and a normalization of the vasopressor activity of angiotensin II (406, 407). GHD induces a clear exacerbation of ischemic tissue damage during low-flow ischemia and reperfusion in rats. This worsening could be, at least partially, due to the reduced release of prostacyclin during the preischemic phase (particularly during reperfusion) and to the enhanced responsivity of coronary smooth muscles to angiotensin II, which increases the coronary artery resistance during reperfusion (406, 407).

Similar results were observed in aged rats in which hexarelin pretreatment achieved a strong protection against myocardial stunning (408). Complete recovery of the cardiac function was present on reperfusion, and the simultaneous reduction of creatine-kinase levels testifies to the integrity of myocardial cell membranes and the preservation from the contractile impairment that follows oxygen readmission (408).

Evidence that GHS activities are GH-independent and mediated by direct activation of specific myocardial receptors (24, 167, 408) came from a study showing cardioprotective effects in hypophysectomized rats (409). Moreover, in an isolated blood-perfused rabbit heart model of stunning, in which brief ischemia- and reperfusion-induced functional impairment without detectable necrosis or apoptosis, 14-d pretreatment with GHRP-2, but not with recombinant human GH, has been reported to selectively protect against the diastolic component of the postischemia-reperfusion myocardial dysfunction (410). Such an improvement in diastolic stiffness was independent of coronary blood flow and of serum GH and IGF-I levels.

Hexarelin increases stroke volume and cardiac output and decreases total peripheral resistance in a rat model 4 wk after experimental myocardial infarction induced by ligation of the left coronary artery (411). A positive effect of GHS on cardiac contractility was also seen in an isoproterenol-induced rapid-pacing porcine model of heart failure in which 3-wk treatment with CP-424,391, an orally active GHS, improved left ventricular fractional shortening (412). Although the mechanisms underlying the inotropic activity of synthetic GHS are still unclear, there is evidence that they increase papillary muscle contractility via actions on endothelial cells and/or nerve endings (413).

It has to be noticed that ghrelin does not share all the cardiovascular actions of synthetic GHS. Ghrelin negligibly protects the heart from ischemia in rats (414), suggesting that the effects of synthetic GHS occur via binding and activation of binding sites specific for peptidyl GHS (25, 26). The inactivity of ghrelin agrees with the existence of a receptor specific for peptidyl GHS only (25, 26, 49, 167, 415). This receptor has a molecular mass that is higher (84 kDa) than that of GHS-R 1a and shows no homology with this receptor; its predicted amino acid sequence is similar to CD36, a multifunctional receptor also known as glycoprotein IV, which would therefore mediate the coronaric actions of peptidyl GHS (49).

Although probably inactive at the coronary level, ghrelin possesses other cardiovascular activities. Chronic administration of ghrelin is able to improve cardiac contractility in GHD rats and even in rats with chronic heart failure (416) in which it also attenuates the development of left ventricular remodeling and cardiac cachexia. Moreover, both in chronic heart failure and in hypophysectomized rats, prolonged administration of ghrelin is associated with a reduction of systemic vascular resistance, probably reflecting a decrease of the afterload, more than a direct myocardial effect (416).

Interestingly, hexarelin, acylated ghrelin, and even nonacylated ghrelin are able to prevent cell death of cultured H9c2 cardiomyocytes and endothelial cells, induced by doxorubicin, serum withdrawal, or activation by FAS-ligand (170, 171). These molecules probably stimulate intracellular signaling pathways involved in the process of survival in cultured cardiomyocytes, including tyrosine phosphorylation of intracellular proteins and activation of extracellular-signal-regulated kinase-1 and -2 and protein kinase B/AKT (170). Because nonacylated ghrelin is unable to activate the GHS-R 1a (17), these data further indicate existence of another cardiac GHS-R subtype. These data also indicate that nonacylated ghrelin has at least some biological activities (8).

Studies in healthy volunteers indicated that synthetic GHS administration induces a prompt increase in left ventricular ejection fraction (417). The same effects have been demonstrated in hypopituitary adult patients with severe GHD (417, 418). As in normal subjects and in GHD patients, the synthetic GHS-induced increase in left ventricular ejection fraction occurred without variations in left ventricular end diastolic volume, mean blood pressure, or heart rate, even in

patients with postischemic dilated cardiomyopathy (417–421). More recently, in patients with coronary artery disease undergoing bypass surgery, acute administration of hexarelin increased left ventricular ejection fraction, cardiac index, and cardiac output (422). Like in animals, the mechanisms responsible for these inotropic activities of synthetic GHS are unclear.

In humans, ghrelin and GHS possess cardiovascular activities. In fact, its administration in normal subjects and even in patients with chronic heart failure significantly decreases systemic vascular resistance and increases cardiac index and stroke volume index (38, 423). This is accompanied by a concomitant reduction in mean arterial pressure, but not by any change in heart rate, mean pulmonary arterial pressure, or pulmonary capillary wedge pressure. Despite its hypotensive effect, which is independent of the GH-IGF-I and nitric oxide system (50), ghrelin slightly increased epinephrine but not norepinephrine levels (38, 164). The pathophysiological significance of these phenomena is presently unknown.

3. Modulation of proliferation of neoplastic cells. Specific binding sites for peptidyl and nonpeptidyl GHS are present in normal and neoplastic human thyroid tissue. Binding sites for GHS have been demonstrated in all follicular and most parafollicular thyroid carcinomas, as well as in different human thyroid tumor cell lines (follicular, papillary, and anaplastic carcinoma cell lines). Moreover, medullary, but not follicular, thyroid carcinomas and carcinoma cell lines remarkably express ghrelin. Ghrelin and peptidyl and nonpeptidyl GHS inhibit [^{3}H]thymidine incorporation and cell proliferation in all thyroid tumor cell lines, already within 24 h (129, 424, 425). Interestingly, the same actions are shared by nonacylated ghrelin that are also particularly accumulated in human medullary thyroid carcinomas (129).

GHS-Rs are found in tumoral tissues from organs that do not express these receptors in physiological conditions, such as mammary gland tissue (27). The presence of specific GHS-Rs was shown in breast cancer, but not in fibroadenomas or normal mammary parenchyma. In breast tumors, the highest binding activity is present in well-differentiated invasive breast carcinomas and is progressively reduced in moderately to poorly differentiated tumors. GHS-Rs are also present in both estrogen-dependent (MCF7, T47D) and estrogen-independent (MDA-MB231) breast cancer cell lines, in which ghrelin and synthetic GHS cause inhibition of cell proliferation. Like in the cardiovascular system and in thyroid carcinomas, the same effect of acylated ghrelin is shared by the nonacylated molecules, again indicating that nonacylated ghrelin is a biologically active peptide possessing at least antiproliferative actions (27). Because nonacylated ghrelin is generally unable to bind the GHS-R 1a, these data indicate the possibility that the antiproliferative effects of acylated and nonacylated ghrelin on breast and thyroid cancer cells are mediated via a GHS-R subtype that is different from the GHS-R 1a (21).

Other data indicate that neuroendocrine carcinoid tumors (26, 121, 128, 163, 166, 169, 426) and even adenocarcinomas of the lung express specific GHS binding sites (426). These sites are also present in the human lung cancer cell line CALU-1, of which the proliferation is inhibited by synthetic peptidyl GHS and analogs such as the EP-80317, but not by ghrelin (426).

The GHS-R 1a and 1b, as well as ghrelin, are also expressed in all prostate cancer cell lines. The PC-3 prostate cancer cell line, however, showed increased cell proliferation *in vitro* after exposure to ghrelin, suggesting that autocrine-paracrine pathways involving ghrelin might be capable of stimulating cell proliferation (46). Indeed, ghrelin-induced cell proliferation was also found in a hepatoma cell line, the H9c2 cardiomyocyte cell line, and rat adrenal cortex (401, 427, 428). Despite the potential proliferative effect on some neoplastic cell types, ghrelin is currently investigated as an anticachectic agent in tumor-bearing animal models, where it seems to exert anabolic actions (429, 430).

VI. Ghrelin as an Important Member of the Survival Kit of Nature

Ghrelin biology is very well preserved, possibly indicating an important role for this peptide hormone, which was discovered no more than 4 yr ago. Apart from humans, rats, and mice, ghrelin expression and/or its biological actions have been demonstrated in many species (43, 54, 135, 244, 324, 431–438).

If ghrelin plays an important biological role in each of these species, what is this role? Although new aspects of this fascinating hormone are discovered nearly on a daily basis, it seems to be reasonable that ghrelin represents a crucial endocrine link connecting physiological processes regulating nutrition, body composition, and growth (51). Based on current knowledge, we speculate that ghrelin ensures that sufficient amounts of energy are available for GH to stimulate growth and repair (439). Assuming the induction of a positive energy balance to be one of ghrelin's most powerful physiological functions, from a teleological point of view, it appears that ghrelin signals the brain when energy must be consumed or stored (51). The observed physiological actions of ghrelin appear to be in accordance with this hypothesis: ghrelin increases food intake (33), decreases fat oxidation (33), and suppresses body core temperature (440).

This function, which may have been developed by evolutionary selection processes for survival in times of reduced caloric supply, now still promotes a positive energy balance, although obesity, and not starvation-induced cachexia, is today's predominant nutrition-related disorder in industrialized countries (441). Recent linkage studies have shown that rare polymorphisms of the preproghrelin gene might protect against obesity-related symptoms (442), possibly indicating that a signaling mechanism once developed to prolong life now may have turned into a health hazard in the presence of changed environmental conditions such as a palatable high-caloric food supply and an increasingly sedentary lifestyle (443).

Several recent studies show that circulating ghrelin peptide levels are decreased in obese individuals (202, 219, 444, 445) as well as postprandially (242), whereas ghrelin levels are increased under cachectic and anorectic conditions (245, 416, 446, 447), as well as during food deprivation (33, 243,

245) or preprandially (209). This negative association between ghrelin concentrations and acute feeding on one hand and chronic positive energy balance on the other is interpreted by many as an adaptive physiological response (51), which, at least in the case of obesity, fails to reestablish an appropriate energy balance (242). That further reduction of the already low plasma ghrelin concentrations in obese individuals could possibly still trigger the reduction of body fat mass, or at least prevent recidivism to obesity after diet-induced weight loss, has recently been shown by Cummings *et al*. (202). After gastric bypass surgery, obese patients exhibited a more impressive weight loss than can generally be achieved by voluntary caloric restriction. Cummings *et al*. (202) found that gastric bypass surgery is associated with low circulating ghrelin concentrations close to the detection limit, whereas weight loss induced by voluntary caloric restriction is coupled to elevated circulating ghrelin concentrations, most likely triggering increased hunger and decreased fat oxidation. Although the hypothesis that these adaptive ghrelin responses to caloric restriction may contribute to the obesity epidemic via increased recidivism is intriguing, only the availability of a potent ghrelin antagonist will make it possible to test whether a pharmacological simulation of gastric bypass surgery could represent an effective treatment option for obesity.

A single exception to the general observation of adaptive changes in ghrelin secretion in response to acute or chronic changes in energy stores is adding further evidence to solidify the theory that ghrelin is a clinically relevant stimulator of hunger and adiposity. Patients with PWS not only suffer from uncontrollable hunger, increased fat mass, and physical hypoactivity, but also have severalfold increased plasma ghrelin levels, when compared with body mass index of matched healthy controls (402, 448, 449). All other known obese states are associated with low plasma ghrelin levels, including monogenic causes of obesity (360). Although the genetic etiology of PWS does not offer a pathogenetic role for ghrelin, indirect mechanisms such as chronic hypoinsulinemia or GHD could be responsible for hyperghrelinemia and its respective consequences (449). Again, only an effective ghrelin antagonist will allow for testing this hypothesis and will possibly represent a much-needed therapeutic option for these patients.

The mechanisms behind adaptive adjustment of ghrelin secretion to stored fat mass clearly require adipocyte-derived signals to communicate the level of stored fat to ghrelin-secreting A cells in the gastrointestinal tract. Recent studies in leptin receptor mutant Zucker rats, leptin receptor-deficient db/db mice, and leptin-deficient ob/ob mice suggest that leptin might not be that signal, because ghrelin levels in these obese rats are low compared with lean matched controls, indicating that the activation of the leptin receptor at the levels of the ghrelin-secreting cell is not the crucial signal and that other adipokines could be responsible for decreased ghrelin levels in obesity (246). Although it has been established quite well that circulating ghrelin concentrations under several conditions presenting with low body fat mass, as for example in cancer cachexia (447), anorexia nervosa (446), or cardiac cachexia (416), are significantly elevated, the existence of a putative ghrelin resistance syndrome under these conditions has not yet been established. It seems possible, however, that a decreased ghrelin responsiveness to constant exposure of ghrelin may be realized similar to leptin resistance syndrome postulated in hyperleptinemic obese patients (450). If this scenario can be proven, it might be difficult to establish ghrelin or one of its receptor agonists as a treatment for anorexia or cachexia, although high doses might still show a favorable effect. A comparable situation occurs in type 2 diabetes, in which insulin treatment can still be useful although insulin levels are already increased in response to insulin resistance (451).

In summary, ghrelin as part of the survival kit of nature might be responsible for synchronizing growth regulation with energy balance to make certain that energy is available for GH actions during growth and repair. Under today's highly palatable and abundant diet environmental conditions, however, the orexigenic and adipogenic drive of ghrelin may be turning this hormone into a health hazard (452).

VII. Pharmacological and Clinical Perspectives

Because GH is a large protein that must be administered by injection or inhalation, ghrelin agonists (and/or GHS) have offered promise for a more convenient and socially acceptable oral delivery of an agent that stimulates endogenous GH. Indeed, it was that potential that drove Merck & Co. to lead a search for an orally available GHS (453). Others realized enormous potential for the much more potent GHRPs, such as GHRP-2 (454) and hexarelin (20), despite their relatively poor bioavailability (455). General clinical utility for these ghrelin mimics as GH therapeutics was reviewed by many (20, 453, 456–458). These discussions focused on uses in pediatrics (459) and geriatrics (72, 318, 460, 461) and on uses to combat the catabolism accompanying critical illness (462). Clinical investigators have also exploited the ability of ghrelin agonists to release GH by mechanisms distinct from GHRH as a diagnostic tool (463–466). Interestingly, the GHRPs (49, 420, 422, 467) and perhaps ghrelin (50) may be used to treat heart failure and hypertension. Such indications have not been made for the nonpeptide agonists of ghrelin, which may be a result of their lower affinity for the GHS-R 1a or because the peptides bind to a yet unidentified receptor. Ghrelin receptors were recently identified in human cancer (27, 424), ghrelin agonists may have antiproliferative actions (426), and in some cancers ghrelin antagonists may be indicated.

Until ghrelin was exposed as a hormone that stimulates appetite in rodents and humans, there was no demanding but unmet clinical need for a ghrelin antagonist. Although such a molecule may be expected to reduce GH levels and thus could be indicated for the treatment of acromegaly, other effective agents are available. The new possibility of using a ghrelin antagonist for the treatment of obesity initiated a scramble by many groups to hunt for such a valuable agent. All groups that had efforts to discover and study GHS have the tools and likely lead molecules to begin this pursuit. Thus, it will not be long before antagonists are disclosed and tested for the treatment of obesity. It is conceivable that initial trials may include obese PWS patients (402).

Acknowledgments

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