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Ghrelin in Health and Disease

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Roy G. Smith • Michael O. Thorner
Editors

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Editors
Roy G. Smith
Department of Metabolism and Aging
The Scripps Research Institute Florida
Jupiter, FL, USA

Michael O. Thorner
Department of Medicine
University of Virginia
Charlottesville, VA, USA

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Preface

The past 5–10 years has seen an explosion of publications on the pharmacological properties of ghrelin and the implications of how this information might extrapolate to understanding ghrelin's role in normal physiology. The pharmacological studies prompted evaluation of the potential benefits of ghrelin as a therapeutic agent in a plethora of disease states. Ghrelin treatment appears to be very well tolerated and the results are encouraging. For chronic use the short half-life and poor oral bioavailability of ghrelin will continue to be a major limitation; however, once clinical targets are identified the availability of propriety long-acting oral ghrelin mimetics developed prior to the discovery of ghrelin, but shelved, will likely emerge as clinical candidates. This book reveals the intriguing properties of ghrelin and its receptor (GHSR1a) with a clinical bias on diseases that may benefit from treatment with ghrelin receptor agonists or antagonists. Each chapter highlights current work in specific areas with the overall objective of stimulating the reader's creativity, irrespective of medical or scientific speciality, towards new ideas of unmet medical needs that may benefit from targeting ghrelin signaling pathways.

The first chapter provides a historical perspective of the discovery of ghrelin beginning in the early 1980s with the identification of a family of synthetic growth hormone (GH) releasing peptides, epitomized by GHRP-6, by Bowers and Momany. Elucidation of the mechanism of action of GHRP-6 led to the development of orally active non-peptide mimetics at Merck Research Laboratories, called growth hormone secretagogues (GHSs), that were exemplified by the long-acting orally active MK0677. In the clinic, MK0677 restored and sustained the amplitude of episodic GH release in elderly subjects to match the profile observed in young adults. In 1996, MK0677 was used to expression clone an orphan G-protein coupled receptor (GPCR) mediating the action of MK0677 and GHRP6; the receptor was appropriately named the growth hormone secretagogue receptor (GHSR1a). Three years later a team led by Kojima and Kangawa exploited GHSR1a in a cell-based assay to isolate a GHSR1a agonist from stomach extracts; they named the new hormone ghrelin. Although ghrelin was subsequently found to control hypothalamic circuits that increase food intake and was believed to be a hormone driving obesity, more recent evidence question this conclusion in favor of an important role in regulating blood glucose during starvation.

Chemically, ghrelin is an octanoylated 28-amino acid peptide and is unique because its biological activity is dependent upon acylation of serine-3 by a medium-chain fatty acid. Surprisingly, rather than coming from endogenous sources the source of the fatty acid is the diet. The pathway through which the ghrelin precursor is converted to the mature acylated ghrelin peptide is reviewed in Chap. 2. Besides production in the oxyntic mucosa of stomach, ghrelin is also made in pancreatic islets. Based on GHSR1a canonical signaling through phospholipase C ghrelin should increase glucose-stimulated insulin secretion (GSIS); instead, ghrelin inhibits GSIS from islet β-cells by a mechanism involving noncanonical GHSR1a G-protein coupling. Indeed, blocking ghrelin signaling in the islet augments GSIS and prevents impaired glucose tolerance in obese diabetic mice. The basis for the potential application of GHSR1a antagonists in the treatment of type 2 diabetes is presented in Chap. 3. In the stomach ghrelin acts locally as a paracrine hormone that modulates the action of gastrointestinal hormones controlling gastric emptying, motility, and energy balance (Chap. 4). This property has important implications for the therapeutic use of ghrelin and ghrelin mimetics for relieving symptoms of gastroparesis.

Ghrelin increases appetite through neural circuits within the hypothalamus and the brainstem. In the hypothalamus ghrelin activates adenosine 5′ monophosphate-activated protein kinase (AMPK). Intriguingly, evidence for a tight interaction between central ghrelin and appetite stimulation by cannabinoids has emerged where hypothalamic AMPK is a common mediator (Chap. 5). In the arcuate nucleus of the hypothalamus ghrelin activates orexigenic neurons that express neuropeptide Y (NPY) and agouti-related peptide (AgRP). Besides stimulating release of NPY and AGRP, ghrelin also activates inhibitory γ-aminobutyric acid (GABA)-ergic inputs that act on adjacent propiomelanocortin (POMC) neurons suppressing their anorexigenic activity. Hence, reducing activity of POMC neurons augments the stimulatory effects of NPY and AGRP on food intake (Chap. 6).

Ghrelin and des-acyl ghrelin are present in the circulation. The enzyme that acylates the ghrelin peptide, Ghrelin O-Acyltransferase (GOAT), colocalizes with ghrelin-expressing cells in the stomach and duodenum. Following acylation ghrelin moves to the Golgi apparatus, and cleavage by prohormone convertase 1/3 produces the 28-amino acid active peptide (Chap. 7). In spite of extensive searches a receptor for des-acyl ghrelin has not been identified; furthermore, GHSR1a remains the only functional receptor for ghrelin. GHSR1a is expressed in the suprachiasmatic nucleus and areas of the hypothalamus implicating a role in regulation of circadian rhythms, sleep, metabolism, and body temperature. Indeed, evidence is emerging for ghrelin and perhaps additional products of the preproghrelin (Ppg) gene, such as obestatin, behaving as important components of the arousal system (Chap. 8).

GHSR1a is widely expressed in the central nervous system (CNS). The CNS coordinates activities that ensure adaptation and survival of animals. The ability to aquire food and store energy is fundamental; hence, hunger must be a motivational force. Ghrelin remains the only hormone that induces feeding behavior suggesting that it has an important role to ensure survival under adverse conditions. Besides actions on the hypothalamus, ghrelin affects excitatory synaptic transmission in the

hippocampus and modulates activity of dopamine neurons. An important role for ghrelin as a regulator of cognitive function and inhibitor of neurodegeneration is reviewed in Chap. 9. Recent reports also suggest that centrally acting ghrelin stimulates mesolimbic reward circuits; therefore, GHSR1a antagonists that cross the blood–brain barrier may prove beneficial in treating alcoholism and other addictive disorders (Chap. 10).

The development of long-acting orally active mimetics of ghrelin, such as MK0677, was motivated by their potential use for improving quality of life in the elderly. Almost one in five of those aged 80 years is unable to walk. Hence, identifying pharmacologic agents that prevent/reverse frailty and help the rapidly growing elderly population maintain their independence is a major unmet medical need. Chapter 11 reviews the potential benefits of treating the elderly with stable long-acting ghrelin mimetics. The very old in the context of mild chronic inflammation frequently exhibit cachexia—a hypercatabolic state characterized by anorexia, accelerated loss of skeletal muscle, and reduced subcutaneous fat. Cachexia is often present in cancer patients and is exacerbated by chemotherapy. Patients with chronic infections, including AIDS, obstructive pulmonary disease, heart failure, and liver disease succomb to cachexia, which can be a direct cause of death. Although cachexia associated with cancer is accompanied by increased levels of endogenous ghrelin, elevating ghrelin tone further by administering ghrelin or orally active ghrelin mimetics improves appetite and weight gain (Chap. 12). Similarly, ghrelin administration has shown benefit in patients with end-stage renal disease (Chap. 13). Treating the malnutrition, vascular disease, and anorexia with nutritional supplements or dialysis is generally ineffective; however, short-term studies with ghrelin administered subcutaneously show promise (Chap. 13). Should these benefits be sustained during long-term treatment, either ghrelin or long-acting ghrelin mimetics may improve clinical outcomes in patients with chronic renal failure.

Autoimmune diseases are also potential therapeutic targets for ghrelin and its mimetics. The anti-inflammatory properties of ghrelin in animal models of sepsis, and arthritis show that ghrelin enhances survival and is protective. Anecdotally, patients with autoimmune diseases such as multiple sclerosis (MS) or rheumatoid arthritis (RA) claim their symptoms are reduced by fasting. During fasting, circulating ghrelin concentrations increase and leptin levels decrease and both hormones modulate immune responses. Studies on helper T (Th) cell subsets indicate that Th1 cells produce cytokines that contribute to the pathogenesis of autoimmunity, whereas Th2 cells and regulatory T cells (Treg) are protective. Starvation leads to Th2 bias. Low leptin modulates Th1/Th2 balance in favor of Th2, consistent with symptomatic relief reported during fasting. The chronic inflammatory demyelinating disease of the CNS, muscular cclerosis (MS), is a disabling neurological disorder in young adults where ghrelin may have therapeutic benefit. For example, in a commonly used animal model of MS, experimental autoimmune encephalomyelitis (EAE), ghrelin treatment suppressed the clinical signs of EAE and ameliorated the clinical course of the disease (Chap. 14).

The final chapter reviews a fascinating partnership within the brain of neurons that co-express the ghrelin receptor (GHSR1a) and dopamine receptor subtype-2

(DRD2). In these neurons, a DRD2 agonist suppresses appetite dependent upon the ghrelin receptor, but not ghrelin, by a mechanism involving formation of GHSR1a:DRD2 heteromers. The formation of heteromers allosterically modifies DRD2 signaling resulting in dopamine-induced anorexia. Furthermore, the results resolve the paradox of a physiological function for GHSR1a in the brain where endogenous ghrelin is absent. By selectively targeting GHSR1a:DRD2 heteromers, more specific therapeutic interventions for obesity and disorders of dopamine signaling such as schizophrenia and post traumatic stress disorder may be found. High selectivity is obtained by selectively blocking dopamine action in GHSR1a:DRD2 expressing neurons with a GHSR1a antagonist, without affecting neurons expressing DRD2 alone. This approach should also improve the side-effect profile associated with the use of DRD2 antagonists in treating psychiatric disorders that block dopamine signaling in all DRD2 expressing neurons.

Jupiter, FL, USA — Roy G. Smith
Charlottesville, VA, USA — Michael O. Thorner

Contents

Contributors

Omar Al-Massadi, M.D. Department of Medicine, Instituto Salud Carlos III, Complejo Hospitalario Universitario de Santiago (CHUS), CIBER de Fisiopatologia Obesidad y Nutricion (CB06/03), Santiago de Compostela University, Santiago de Compostela, Spain

Zane B. Andrews, Ph.D. Department of Physiology, Monash University, Clayton, VIC, Australia

Damien Ashby, MRCP, Ph.D. Hammersmith Hospital, Imperial College Kidney and Transplant Institute, London, UK

Stephen Bloom, M.D., D.Sc., F.Med.Sci Department of Endocrinology, Imperial College Healthcare NHS Trust, London, UK

Boldbaatar Damdindorj, M.D., Ph.D. Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, Shimotsuke, Japan

Cyril Y. Bowers, M.D. Department of Medicine, Endocrinology/Peptide Research, Tulane University Health Sciences Center, New Orleans, LA, USA

Felipe F. Casanueva, M.D., Ph.D. Department of Endocrinology and Nutrition, Universitario de Santiago de Compostela, Santiago de Compostela, Spain

Peter Choi, M.A., M.B., BChir, Ph.D., FRCP Imperial College Healthcare NHS Trust, London, UK

Michael Alexander Cowley, Ph.D. Department of Physiology, Monash University, Clayton, VIC, Australia

Ana Belen Crujeiras, M.D. Department of Medicine, Complejo Hospitalario Universitario de Santiago (CHUS), Santiago de Compostela University, Santiago de Compostela, Spain

Katsuya Dezaki, Ph.D. Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, Shimotsuke, Japan

Suzanne L. Dickson, B.Sc., Ph.D. The Sahlgrenska Academy at the University of Gothenburg, Institute of Neuroscience and Physiology, Gothenburg, Sweden

Emil Egecioglu, Ph.D., M.Sc. Department of Physiology, University of Gothenburg, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, Gothenburg, Sweden

Jörgen Engel, Ph.D., M.D. Department of Pharmacology, University of Gothenburg, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, Gothenburg, Sweden

Pablo J. Enriori, Ph.D. Department of Physiology, Monash University, Clayton, VIC, Australia

Basil Burney, M.D. Department of Endocrinology, Michael E. DeBakey VA Medical Center, Houston, TX, USA

Jose M. Garcia, M.D., Ph.D. Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center, Baylor College of Medicine, Houston, TX, USA

Ezio Ghigo, M.D. Division of Endocrinology, Diabetology and Metabolism, Molinette Hospital, University of Torino, Torino, Italy

Riccarda Granata, Ph.D. Division of Endocrinology, Diabetology and Metabolism, Molinette Hospital, University of Torino, Torino, Italy

Tamas L. Horvath, D.V.M., Ph.D. Yale Program in Integrative Cell Signaling and Neurobiology of Metabolism, Section of Comparative Medicine, Yale University School of Medicine, New Haven, CT, USA

Elisabet Jerlhag, Ph.D. Department of Pharmacology, University of Gothenburg, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, Gothenburg, Sweden

Kenji Kangawa, Ph.D. National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan

Levente Kapás, M.D., Ph.D. WWAMI Medical Education Program, Washington State University, Spokane, WA, USA
Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA, USA

Andras Kern, Ph.D. Department of Metabolism and Aging, The Scripps Research Institute, Jupiter, FL, USA

Henriette Kirchner, M.S., Ph.D. Department of Internal Medicine and Endocrinology, Metabolic Diseases Institute, University of Cincinnati, Cincinnati, OH, USA

Masayasu Kojima, M.D., Ph.D. Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Fukuoka, Japan

Blerina Kola, M.D., Ph.D. Centre for Endocrinology, William Harvey Research Institute, Barts and The London, Queen Mary‘s School of Medicine and Dentistry, London, UK

Márta Korbonits, M.D., Ph.D. Centre for Endocrinology, William Harvey Research Institute, Barts and The London, Queen Mary‘s School of Medicine and Dentistry, London, UK

Tomoyuki Kurashina, M.D. Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, Shimotsuke, Japan

Aart J. van der Lely, M.D., Ph.D. Department of Internal Medicine, Erasmus University MC, Rotterdam, The Netherlands

Sachiko Miyake, M.D., Ph.D. Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan

Ralf Nass, M.D. Department of Internal Medicine, Division of Endocrinology, University of Virginia, University Hospital, Charlottesville, VA, USA

S.J.C.M.M. Neggers, M.D., Ph.D. Section of Endocrinology, Department of Medicine, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands

G.A. Reynolds, B.A. Department of Medicine, Endocrinology/Peptide Research, Tulane University, Health Sciences Center,, New Orleans, LA, USA

Miski Scerif, M.B.B.S., B.Med.Sci. Centre for Endocrinology, William Harvey Research Institute, Barts and The London, Queen Mary’s School of Medicine and Dentisty, London, UK

Luisa María Seoane, Ph.D. Complejo Hospitalario Universitario de Santiago de Compostela/SERGAS, Santiago de Compostela, Spain

Roy G. Smith, Ph.D. Department of Metabolism and Aging, The Scripps Research Institute Florida, Jupiter, FL, USA

Eva Szentirmai, M.D., Ph.D. WWAMI Medical Education Program, Washington State University, Spokane, WA, USA
Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA, USA

Michael O. Thorner, M.B., B.S., D.Sc. Department of Medicine, University of Virginia, Charlottesville, VA, USA

Jenny Tong, M.D., M.P.H. Department of Medicine, University of Cincinnati, Cincinnati, OH, USA

Matthias Tschöp, M.D. Department of Internal Medicine, University of Cincinnati, Cincinnati, OH, USA

Johannes D. Veldhuis, M.D. Endocrine Research Unit, Mayo Clinic, Rochester, MN, USA

Toshihiko Yada, Ph.D. Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, Shimotsuke, Japan

Takashi Yamamura, M.D., Ph.D. Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan

Chapter 1
Ghrelin: A History of Its Discovery

Cyril Y. Bowers, G.A. Reynolds, and Johannes D. Veldhuis

Abstract Historically, in 1981, growth hormone-releasing peptides (GHRPs) initially were thought to reflect the actions of elusive GHRH but fortunately in 1982 GHRH was isolated. By 1984 GHRP-6 results revealed that the actions of GHRH and GHRP were distinguishable from each other. To appreciate GHRP and GHRH interrelationships on GH release, detailed in vitro and in vivo dose–response studies were essential. Over subsequent years GHRPs were studied by many talented basic and clinical investigators. By 1995 GHRP icv administration was found to increase food intake in conscious rats and in 1996 the GHS-1a receptor was cloned. Finally, in 1999 the GHRP/GHS natural hormone, ghrelin, was isolated, synthesized, and found to have essentially the same activity as that of GHRPs/GHSs in animals and humans. A major surprise and a reorienting finding was the primary anatomical location of ghrelin in the stomach and, in addition, strong enhancement of food intake. Over time, GH secretion has been hypothesized to be primarily regulated by the hypothalamic hypophysiotropic tripartite system of GHRH, ghrelin, and SRIF rather than the bipartite system of GHRH and SRIF. Since the isolation of ghrelin, actions of this hormone have continued to expand from the hypothalamic CNS to peripheral sites. This includes both direct and indirect actions particularly related to nutrition and metabolism as well as a cornucopia of unexpected actions. In summary, unnatural GHRP begot natural ghrelin and its receptor. This reverse pharmacology approach forecasts that variations of the unnatural to natural sequence of events likely will be more frequent, modified, expanded, and refined in the future.

C.Y. Bowers, M.D. (✉) • G.A. Reynolds, B.A.
Department of Medicine, Endocrinology/Peptide Research, Tulane University Health Sciences Center, 1430 Tulane Avenue, Box SL 53, New Orleans, LA 70112, USA
e-mail: cybowers@tulane.edu

J.D. Veldhuis, M.D.
Endocrine Research Unit, Mayo Clinic, Rochester, MN, USA

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Introduction and Background

This is a history in three parts as depicted in Fig. 1.1. Each part is independent as well as interdependent being additive, synergistic, and expanding. The saga has the elements and spirit imparted by a previous editor of Science, the late Stephen Gould in the "The Hedgehog, the Fox and the Magister's Pox." It conveys that the new is inevitable, exciting, and may be historically significant.

Our growth hormone-releasing peptide (GHRP) research evolved from an ambitious basic-chemical program at Tulane Medical School with Andrew Schally to isolate and characterize all the hypothalamic hypophysiotropic hormones (HHH) including GHRH (1962–1979). A second relevant background component in the development of synthetic GHRPs evolved from structure–activity relationship (SAR) studies of TRH/LHRH agonist/antagonist synthetic analogs with Karl Folkers' group starting in 1980. The strategies, approaches, and techniques utilized in these and also the present studies allowed the peptide chemistry and bioactivity to be directly interrelated and to establish a chemical, bioactivity database for designing improved peptides and more indirectly nonpeptide analogs.

Beginning (1974–1994)

In 1974 the Met/Leu twin opiate peptides, TyrGlyGlyPheMetCOOH and TyrGlyGlyPheLeuCOOH, were isolated from brain extracts by John Hughes. Shortly following this achievement, Kang Chang, a former collaborator on TRH and LHRH analogs sent me (C. Bowers) a series of pentapeptide enkephalin analogs he synthesized for pituitary hormone-releasing activity. Because opiates were known to release GH and due to our broader appreciation of the SARs of peptides that released pituitary hormones, these analogs were assessed for various pituitary hormones in vitro. Our first GHRP was the modified enkephalin pentapeptide

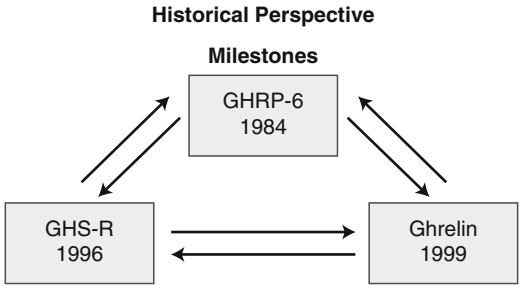

Fig. 1.1 Historical perspective schematic

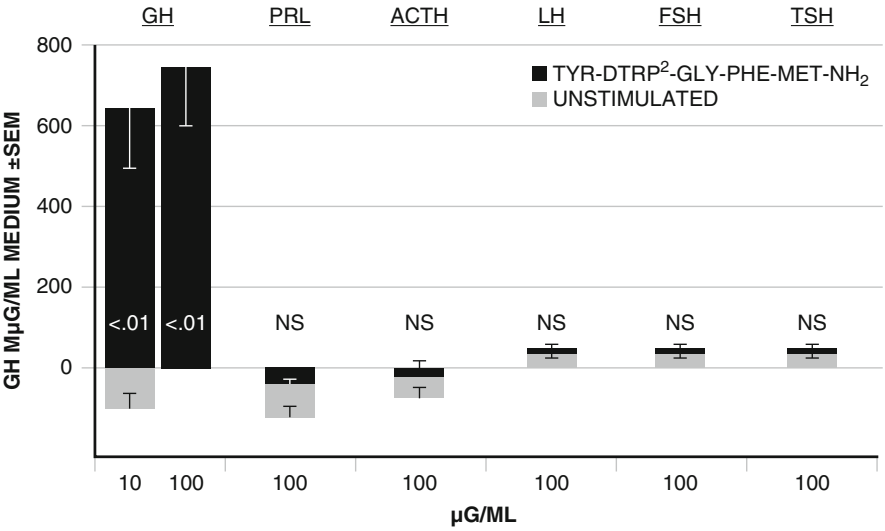

Fig. 1.2 In vitro release of GH by TyrDTrp^{2}GlyPheMetNH$_2$ (DTrp^{2}GHRP). There was no release of any of the other hormones measured. Pituitaries of 20-day-old female rats. Mean of 6 ± SEM. Reproduced with permission from [1]

analog TyrDTrpGlyPheMetNH$_2$ (DTrp^2-GHRP) which released GH by a direct pituitary action and was a sine qua non of HHH. Neither of the natural opiate peptides Met nor Leu enkephalin without C-terminal amidation released GH by a direct pituitary action in vitro. Although the DTrp^2 enkephalin analog activity was low in potency, it only released GH and not TSH, LH, PRL, or ACTH via a direct pituitary site of action as shown in Fig. 1.2 [1]. The presence, number, position, and chirality of the Trp^2 substitution in the first DTrp^2 pentapeptides were important SAR information. Starting with the GH-releasing Met enkephalin pentapeptide, analogs were synthesized which had increasing GH-releasing activity. They included not only DTrp^2 but also DTrp^3, DTrp^{2,3}, and DTrp^2 spacer LTrp^4 analogs. The first three types initially had only in vitro activity while the fourth type pentapeptide, DTrp^2 spacer LTrp^4, was active in vitro and in vivo but the potency was low. In contrast to the natural Met/Leu peptides, all of these analogs were C-terminal amidated pentapeptides [2, 3].

Between 1976 and 1980, more than 600 small synthetic peptides were synthesized and in dose–response studies assayed in vitro for stimulation and inhibition of pituitary hormone(s) release. In these studies stimulation of GH, TSH, LH, FSH, PRL, and ACTH was determined as well as inhibition of the stimulated release of GH by DTrp^2 GHRP, TSH release by TRH, and LH/FSH release by LHRH. This became an increasingly valuable SAR bioinformatic multidimensional pituitary database. The synthesis of the peptides was performed by Kang Chang's group**.** Eventually highly active GHRPs with in vitro and in vivo activity included the four different basic chemical templates designated DTrp^2, DTrp^3, DTrp^{2,3}, and DTrp^2 spacer LTrp^4.

Between 1980 and 1984 what became apparent to the success of developing potent peptide GHRPs was the initial finding that the Met enkephalin analog, $DTrp^2$-GHRP, specifically released GH by a direct pituitary site of action. This allowed effective utilization of past empirical peptide bioactive designs and approaches developed with Karl Folkers' group for LHRH receptor antagonists, development of more potent GHRPs, as well as utilization and incorporation of the theoretical conformational energy approach developed by Momany as a component of the design process. Examples of the Momany conformational approach were published in 1981 on the GHRP pentapeptides and in 1984 on the GHRP hexapeptides [4, 5].

In 1981, a detailed series of in vitro studies were performed on pituitaries of immature versus mature rats. They included effects of $DTrp^2$-GHRP, $DTrp^3$-GHRP, SRIF, PGE_1, theophylline, glucose, and insulin on stimulation and inhibition of GH release. Selective effects were revealed to be a function of the pituitary of mature versus immature rats [3]. For example, the GH stimulatory release definitely was more sustained and also inhibition of the stimulated release was more readily reversible from the pituitary of immature versus mature rats.

In 1982 the hexapeptide, $HisDTrpAlaTrpDPheLysNH_2$, designated GHRP-6, was synthesized which had high potency in specifically releasing GH from the rat pituitary in vitro and also in vivo. A brief summary was published as an abstract in 1982 and subsequently in Endocrinology [6–8]. GHRP-6 significantly released GH in vitro from the pituitary of immature female rats at 1 and 3 ng/ml incubation medium. In 21-day-old female rats, GH (ng/ml serum) acutely released by 1, 10, and 100 μg was 12 ± 4 (<0.02), 151 ± 56 (<0.02), and 381 ± 61. Additionally, the peptide significantly ($<.001$) augmented the body weight gain of 16-day-old female rats by 17.5–10% after 9 and 25 days treatment with 30–100 μg once or twice daily. GHRP did not release LH, FSH, TSH, or PRL in vitro or in vivo. SRIF and SRIF 1–28 inhibited the GHRP GH levels in vivo and in vitro (Table 1.1) [7]. Also, GH levels rose acutely 10- to 25-fold in 2–10 min in rhesus monkeys, lambs, and calves. From the bioactivity and the qualitative interrelated activity among the various GHRPs, it was postulated that they were probably acting on the same receptor.

Since the long-term efforts to isolate the putative natural hypothalamic GHRH still had been unsuccessful and GHRP-6 had the presumed functional attributes of natural GHRH, GHRP-6 was envisioned to be a GHRH analog and endogenous GHRH was envisioned to be a small peptide like TRH, LHRH, or Met/Leu enkephalin. In spite of the variable peptide sequences, the GH release was much more uniform. Their peptide sequences did not readily indicate whether they would be only active in vitro or both in vitro and in vivo. Nevertheless from the bioactivity data, they probably were acting on the same receptor and, thus, the qualitative activity among the various GHRPs could be interrelated. Also, in 1981 and 1982 while pursuing the objective of higher potency GHRP analogs, our first GHRP antagonist was revealed. Substitution of DLys in position 3, $HisDTrpDLys^3TrpDPheLysNH_2$, inhibited the GH release of GHRP-6 both in vitro and in vivo [6].

Table 1.1 In vitro dose–response effect of GHRP-6 (A) (upper panel) and in vivo dose–response effect of GHRP-6 (lower panel)

			GH[a]	
Exp	Peptide	Dose (ng/ml medium)	Δ ng/ml medium ± SEM	*P* vs. –
1	–		−167 ± 114	–
	A	1	955 ± 272	<0.01
	A	3	1,603 ± 305	<0.001
	A	10	2,244 ± 173	<0.001
	A	30	2,184 ± 358	<0.001
2	–		108 ± 77	<0.001
	A	30	1,602 ± 230	–
	A + SRIF-14	30 + 30	912 ± 137	~0.02
	A + SRIF-14	30 + 100	951 ± 64	0.02
	A + SRIF-14	30 + 300	18 ± 66	<0.001
3	–		236 ± 180	~0.001
	A	30	1,691 ± 308	–
	A + SRIF-28	30 + 30	622 ± 98	<0.01
	A + SRIF-28	30 + 100	204 ± 120	0.001
	A + SRIF-28	30 + 300	−576 ± 235	<0.001

		GH[b]			
			P		
Peptide	Dose (μg, sc)	ng/ml serum ± SEM	vs. –	vs. A	vs. SRIF
–		1.4 ± 0.5	–	–	
A	3	108 ± 12	<0.001	–	<0.001
A	10	142 ± 29	<0.001	–	NS
A	30	178 ± 34	<0.001	–	NS
A + SRIF-14	3 + 50	32 ± 7.0	~0.001	<0.001	–
A + SRIF-14	10 + 50	135 ± 27	<0.001	NS	–
A + SRIF-14	30 + 50	217 ± 40	<0.001	NS	–
A + SRIF-28	3 + 20	2 ± 0.9	NS	<0.001	~0.001
A + SRIF-28	10 + 20	40 ± 7.0	<0.001	~0.01	<0.01
A + SRIF-28	30 + 20	78 ± 23	<0.01	~0.05	~0.01

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[a]Mean of 9 ± SEM. – denotes group used for *P* value comparisons

[b]Mean of 6 ± SEM. NS, $P > 0.05$

In 1982 a most important and relevant major accomplishment was the isolation and identification of GHRH from two different functionally hormonal secreting pancreatic tumors of patients with acromegaly by the Guillemin group (44 amino acids [aa] C terminal amide linear peptide) and the Vale, Rivier group (linear 40 aa C-terminal COOH peptide) [9, 10]. Both peptides released GH by a direct pituitary action with high potency in humans. Eventually GHRH 44NH_2 was validated as the natural GH-releasing HHH. The brief history by Roger Guillemin on the unsuccessful isolation of GHRH from hypothalamic extracts after prodigious efforts and its facile isolation from functional pancreatic tumors imparts the complexities and dimensions of the GHRH isolation [11]. The latter underscores the significant

value of the informative basic, clinical combined approach including studies in humans on the effects of GHRH 1–40 and 1–44 [12, 13]. Prior to the isolation of GHRH, the early relevant studies of Frohman et al. involved partial purification of a presumed "GHRH" peptide from a pancreatic tumor from a patient with acromegaly [14].

In 1983 in vitro studies of immature and mature rats revealed that GH was markedly released by GHRP-6 without a change of pituitary GH content and little or no change in pituitary cAMP levels. In comparison, PGE_1 and theophylline markedly increased GH release and also pituitary cAMP levels without a change in pituitary GH content [2, 3, 15]. Again, fortuitously, in 1982 Brazeau et al. reported the in vitro effects of GHRH (GRF) on the release of GH and cAMP in pituitary cell culture [16]. Important was the dual parallel effects on both pituitary GH and cAMP in this dose–response study. Notably GHRPs and GHRH markedly released GH but only GHRH increased release of cAMP [17].

Although GHRP-6 had the biological activity of a HHH, comparison of GHRP-6 and GHRH on GH release both in vitro and in vivo between 1982 and 1984 led us to the conclusion that GHRP-6 represented the activity of a different and additional HHH.

Fortuitously and seemingly poetic, the first unnatural highly active GHRP hexapeptide, GHRP-6, and the elusive long-sought endogenous natural GHRH were isolated and identified essentially within the same early 1980 time period. The successful isolation of endogenous GHRH was greatly facilitated by isolation from a functional pancreatic tumor from a patient with acromegaly rather than from hypothalamic extracts. The availability of both GHRH and GHRP-6 allowed direct comparison of the chemistry and function of the two peptides. A relevant series of interrelated publications to the GHRH isolation are recorded in our 1984 publication [8].

The following findings obtained between 1984 and 1989 again supported the GHRP-6/GHRH hypothesis that these different hormones acted on different receptors. In rats, the GH response of repeated GHRP-6 administration was decreased but not that of repeated GHRH administration. The maximal GH response of GHRH in rats was further increased by simultaneous administration with GHRP-6. GHRP-6 did not prevent GHRH binding in the GHRH radioreceptor binding assay. These results strongly supported that GHRP-6 was not an analog of the hormone GHRH but rather an analog of a new HHH also involved in the regulation of GH secretion. The results indicated that a new receptor for this new hormone existed and would be revealed [7].

A significant number of GHRP-6 and GHRH in vivo effects on GH secretion parallel each other and this was the reason for projecting that GHRP-6 could be an analog of GHRH (GRF). Common biological actions of GHRP-6 and GHRH in vivo included the following: SRIF inhibition of the GH response, synergistic release of GH, increased GH response with SRIF antiserum administration, decreased GH response with GHRH antiserum administration, and increased GH response with opiate administration [18].

Nevertheless, it finally became apparent from observations of the actions of GHRP and GHRH on GH release that they were closely interrelated in their actions

both in vitro and in vivo but there were definite differences which were select, major, and meaningful. Initially, the differences included not only a lack of GHRP-6 binding in the GHRH receptor but also a marked increase of cAMP by GHRH but not GHRP-6, and the in vivo synergistic GH release of combined GHRP-6 and GHRH. It became increasingly apparent from the HHH viewpoint that the primary major regulation of GH secretion is not just via the bipartite GHRH and SRIF system but instead the tripartite GHRH, SRIF, and putative GHRP system and that the putative endogenous GHRP hormone was yet to be isolated and identified.

By in vitro studies utilizing perfusion of dispersed rat pituitary cells, Badger et al. in 1984 again demonstrated similarities and differences of GHRP-6 and GHRH (GRF) on GH release [19]. Also complementary in vivo studies of McCormick et al. in 1984 were performed utilizing conscious male rats pretreated with the dopamine beta hydroxylase inhibitor, diethylthiocarbinate, and anti-somatostatin antiserum to inhibit the GH pulsations and SRIF secretion. In both of the above studies GHRP-6 simulated GHRH in producing the persistent increase in GH pulses but the GHRH effect was considerably more effective and the GH pulse shape was different [20]. In the in vitro study of Badger, 7-h continuous administration of GHRH versus GHRP-6 in the beginning induced a single GH pulse but subsequently the GH pulses were eliminated; basal GH levels were less decreased by the infusion of GHRH than by GHRP-6. The authors suggested that GHRP-6 "may stimulate GH release by activating partially the same mechanism by which GHRH (GRF-44) elicits GH secretion or by different mechanisms." Although this projection is most appropriate, other data emphasized that the GHRH GH release resulted from a direct pituitary action while the GHRP action is on both the hypothalamus and pituitary. Depending on the GHRP dose, endogenous GHRH may only be released at high dosages of GHRP. Unfortunately in the Badger et al. and McCormick et al., in vitro and in vivo studies with combined GHRP-6 and GHRH were not evaluated.

In 1985, Sartor et al. revealed that the GHRP-6- and GHRH GH-releasing action utilizing in vitro monolayer cell culture assays plus calcium-blocking agents, CdCl and verapamil, inhibited the GH response of both peptides as well as basal GH release. Also, combined GHRP-6 and GHRH stimulated GH release additively [21, 22]. Two other publications relevant to GHRH and GHRP-6 GH-releasing actions were by Gloria Tannenbaum and Nicholas Ling in 1984/1985 and the Paul Plotsky and Wylie Vale publication in 1985 [23, 24]. In the former, GHRH and SRIF were proposed to generate the ultradian rhythm of GH release and, in the latter, the patterns of GHRH and SRIF were demonstrated in the hypophyseal portal system. Somatostatin was isolated and identified in 1973 by Brazeau et al. [25].

Because of the physiological importance of pulsatile GH secretion there was a special need to reveal and understand possible roles and interactions of GHRPs/GHSs/ghrelin in relationship to the GH effects of the endogenous hormones SRIF and GHRH. In contrast to rats but parallel to dogs, administration of GHRPs/GHSs to humans induced and augmented normal pulsatile GH secretion even after prolonged continuous administration with sc GHRP-2 and oral MK-0677. Furthermore, combined GHRP-2 and GHRH continuous administration at a constant rate also augmented the normal pulsatile secretion of GH in humans.

A number of articles pertinent to pulsatile GH secretion were published between 1990 and1999 and include some of the details about the receptor, molecular mechanisms, and putative GHRP/GHS hormone. At the end of this chapter, the now past dipartite GH secretory regulatory model, i.e., GHRH/SRIF, is presented in terms of the new tripartite GH regulatory model, i.e., GHRH/SRIF/ghrelin.

First-Generation Chemistry, 1980–1993

A series of different types and families of GHS/GHRP receptor agonists and antagonists have been synthesized and studied. The four generations of GHRPs studied at Tulane were as follows: GHRP-6 (HisDTrpAlaTrpDPheLysNH$_2$), GHRP-1 (AlaHisDβNalAlaTrpDPheLysNH$_2$), GHRP-2 (DAlaDβNalAlaTrpDPheLysNH$_2$), and GHRP-3 (αAibDTrpDProDIleArgNH$_2$). Each generation was about three times or more effective in releasing GH than the previous generation. In 1996 Momany and Bowers summarized GHRP-6 conformational studies [26]. GHRP receptor antagonists also were synthesized and studied during this same time period that the above agonists were developed and/or studied. They included DLys^3-GHRP-6, HisDβNalDLysTrpDPheLysNH$_2$, and [DArg^{1}DPhe^{5}DTrp^{7,9}Leu^{11}]-Substance-P, with antagonist activity which subsequently also was found to be an inverse agonist [18, 27–29].

From 1990 to 2000 a number of GHSs were initially developed by Europeptides, Merck, Novo Nordisk, Genentech, Pfizer, Eli Lilly, and Bristol Meyers**.** Particularly important was Merck's nonpeptide designated MK-0677. The overall chemical details of MK-0677 were published by Art Patchett, Roy Smith, and collaborators from 1993 to 2000 (vide infra) [30–32].

Basic Studies

In Vitro/In Vivo Hypothalamic–Pituitary Studies, 1989–2000

Codd, Shu, and Walker of Smith Kline in 1989 published findings in support of the dual hypothalamic–pituitary sites of action [33]. Specific GHRP-6 binding sites were demonstrated at both the hypothalamus and pituitary. Since the binding results of a series of GHRPs with various degrees of activity paralleled each other, they concluded that the results were specific and meaningful.

Also, in 1989 Cheng and Roy Smith et al. of Merck reported a basic series of valuable in vitro findings of GHRP-6 and GHRH and they also concluded that the two peptides acted on different receptors via discrete mechanisms [34]. In addition, they found that the GHRP-6 half maximal and maximal concentrations were 7×10^{-9} and 10^{-7} M, respectively. Neither naloxone nor the GHRH receptor antagonist [NAcTyr^{1}DArg^2]-GHRH 1-29NH$_2$ altered the GHRP-6 GH release while the

GHRP-6 receptor antagonist, DLys^3-GHRP-6, inhibited the GH release of GHRP-6 but not GHRH. Surprisingly maximal concentrations of combined GHRP-6 and GHRH induced a synergistic (threefold) rather than just an additive effect on GH release. GHRP-6 had no effect on intracellular cAMP levels. Pretreatment with 10^{-7} M GHRP-6 for 1 h decreased basal GH release by 30% and, in addition, decreased the subsequent GH response of GHRP-6 but not that of GHRH. In contrast, GHRH pretreatment for 1 h had no effect on the GH release of GHRP-6 or GHRH. While the GHRP-6 desensitization effect was completely reversed within 1 h after removal of GHRP-6.

The studies of Ross Clark and Ian Robinson et al. in 1989 in conscious male rats were fundamental in further understanding the relationships of GHRP and GHRH on GH release [35]. The following is their conclusion of these studies. "In the conscious animal, GHRP may release GH by complex actions at both a hypothalamic and pituitary level." These important results again underscore the complexities and subtleties of the complicated interrelationships between GHRH and GHRP. During this time period evidence does support both a hypothalamic and pituitary site of action to enhance GH release. Yet to be appreciated is whether the "primary" effect is on the pituitary or hypothalamus.

Complementary to the GHRH + GHRP-6 dual hypothalamic–pituitary actions and synergism on GH release was our series of in vitro/in vivo studies published in 1991 on combined GHRP-6 and GHRH as well as the effects of opiates, GHRH antiserum, SRIF antiserum, and DLys^3-GHRP-6 receptor antagonist [18]. The following is a summary of these results. GHRP releases GH by a unique and complementary dual site of action on the hypothalamus and pituitary. These effects are mediated via non-GHRH and non-opiate hormones or peptides, or their receptors. Also direct prevention of SRIF inhibition of GHRH + GHRP-6 did not explain the combined synergistic release of GH.

A major conclusion in 1991, and also presently, was that GHRP-6 releases an unknown factor (U-factor) which interacts in combination with GHRH on the pituitary to release GH synergistically [18]. The pituitary action of U-factor depends on the interaction of GHRH because GHRH antiserum inhibits the synergistic response of GHRH and GHRP-6. The effect of U-factor itself on GH release is considered to be minimal because GHRP-6 presumably released U-factor after GHRH antiserum pretreatment and yet GH release was low. Primarily U-factor's action could be to augment the effect of GHRH on the pituitary somatotrophs. The action of GHRH is necessary but not sufficient alone to explain the complete scope of the synergistic release of GH when GHRH and GHRP-6 are administered together.

From specific experimental results, we concluded that the dramatic synergistic release of GH by combined GHRP-6 and GHRH in vivo is not explained by a direct pituitary action of the individual peptides. In three different in vitro pituitary assay systems (incubate, perifusion, cell culture) these two peptides nearly always released GH additively. When the GH was released synergistically it ranged only 15–45% greater. In animals and humans, this combined GHRP-6 and GHRH synergism was many fold greater. Additionally, combined activation of the phospholipase C, inositol (1,4,5)-triphosphate (IP_3), protein kinase C, and cAMP intracellular pathways by

GHRP-6 and GHRH unlikely explain the synergism of the two peptides in vitro as in the studies of Cheng et al. [34]. GHRP-6 and GHRH are well established to activate these two intracellular pathways and yet in three different in vitro systems these two peptides in our studies essentially released GH additively.

Pituitary Studies, 1980–1996

The results recorded in Table 1.1 utilizing rat pituitaries in vitro and 21-day-old rats in vivo indicate that lower dosages of SRIF-28 were more active than SRIF-14 in both assays in inhibiting the GH response of GHRP-6 [7].

In vitro electrophysiological studies using single rat somatotroph cells have provided information about the effects of GHRP/GHS on plasma membrane ionic currents and peripheral membrane potentials [36].

Whether GHRP/GHS-induced depolarization of somatotrophs is mediated via Na^+ or K^+ channels remains to be elucidated. Substantial evidence indicates that GHRP/GHS depolarizes somatotrophs during which voltage-dependent L-type Ca^{2+} channels are opened and Ca^{2+} levels are elevated. Herrington and Hille demonstrated that the transient but not the persistent elevation of Ca^{2+} induced by GHRP is still elicited in the presence of 100 nM somatostatin [37].

Indirect and direct evidence obtained from in vitro studies indicates that GHRH activates phospholipase C, which in turn hydrolyzes membrane phosphatidyl inositol (PI) into IP_3 and diacylglycerol (DAG) [38, 39]. Intracellular free calcium Ca^{2+} is released by PI presumably from the pool of Ca^{2+} stored in the endoplasmic reticulum, whereas DAG activates PKC which in turn phosphorylates intracellular proteins and possibly peripheral cell membrane proteins involved in the exocytosis process that releases GH [40]. The post receptor action of GHRP differs from that of GHRH since GHRH stimulates the adenyl cyclase cAMP protein kinase A signaling pathway.

In contrast to GHRH, GHRP has not been reported to increase GH synthesis in vitro [41]. Of particular importance would be to determine the effects of GHRP with and without GHRH in vitro and in vivo after acute and chronic administration on GH synthesis. If it can be demonstrated that chronic administration of GHRP increases GH synthesis in vivo, it would be important to determine whether this is due to the hypothalamic release of GHRH in response to GHRP and/or to a direct pituitary action of GHRP.

Also pertinent is that during continuous GHRP administration the GH secretory response to GHRP is desensitized at the same time that pulsatile GH secretion is enhanced. Thus concomitant desensitization and sensitization to the action of GHRP occur. It is possible that GHRP is tonically secreted along with somatostatin during the 1.5–2.5-h interpulse interval when GH levels are low. Even though GHRP may not release GH during this interval, it presumably may still actively modulate a molecular mechanism(s) within the somatotrophs. Thus GHRP may produce a cumulative priming effect at the molecular level to facilitate the release of the next GH pulse.

Hypothalamic 1990–1997

The important pioneering studies of Suzanne Dickson in collaboration with Gareth Leng and also Ian Robinson began in 1990–1993 on the indirect and direct actions of GHRP-6 on the hypothalamus of rats [42]. These studies allowed identification of various hypothalamic receptors and/or peptides, locations, and activation in particular such as GHRH, GHRP/GHS, SRIF, and NPY [43, 44]. A special amount of new hypothalamic information was obtained via the utilization of cfos protein changes induced by GHRP/GHS and GHRH in vivo and in vitro [42, 45]. Following iv bolus GHRP-6, arcuate neurons were activated that extended to the median eminence, thus supporting that they were primarily neurosecretory neurons (1993) [42]. By correlating the distribution of the cfos messenger RNA changes it became possible to determine the increases in the responses of GHRP/GHS and the distribution of other peptides normally synthesized by arcuate neurons. The finding revealed that GHRP stimulated one-half of the NPY neurons and one-fifth of the GHRH neurons present in the arcuate nucleus [43]. This same group demonstrated similar hypothalamic effects of GHRP-6 and a Merck nonpeptide GHS (1995) in rats. In 1995 Dickson et al. demonstrated that systemic administration of GHRP-6 increased fos protein expression in the hypothalamic arcuate nucleus of GH-deficient dw/dw rats, hypophysectomized rats, and lit/lit mice [45]. Also, Kamegai demonstrated that GHRP-2 induced cfos expression in the arcuate nucleus [46].

Between 1990 and 1999 the necessity and significant value of direct hypothalamic neurochemical and electrophysiological studies became increasingly apparent in revealing CNS actions of the GHRP/GHS [44, 46]. Electrophysiological studies by Dixon et al. also allowed distinguishing arcuate neurosecretory neurons that extended to the median eminence from arcuate nonsecretory neurons although both were stimulated by GHRP-6 [42, 43, 47].

Results of cloning studies indicated the identity of the pituitary and hypothalamic GHRP/GHS receptor at these two anatomical sites and thus the possible same intracellular signal transduction pathway [48].

Other CNS effects described by Dickson and collaborators extending into 1997 included increase of hypothalamic fos protein expression by both peptide and nonpeptide secretagogues. These results further indicted possible roles of hypothalamic interneurons and also the complexity of GHS actions at the hypothalamic level [43, 44, 46].

An additional notable result was obtained by William Locke et al. in 1995 in which icv GHRP-6 administration increased food intake in rats [49]. By 1996 Okada and Wakabayashi et al. published that icv GHRP-2 also increased food intake in conscious rats [50]. Special aspects of this latter study were the following. Intracerebroventricular (icv) GHRH-increased food intake was inhibited by a GHRH antagonist but this antagonist did not inhibit GHRP-2-induced food intake. Also of special significance was that combined GHRP-2 and GHRH in maximal dosage amplified food intake.

Overview of In Vitro/In Vivo Studies

In 1996 Fairhall, Robinson et al. published a series of erudite and informative major conclusions about the GHRP, GHRH, and SRIF roles and inter relationships in the regulation of GH secretion obtained in the laboratory guinea pig model [51]. The following was concluded. (1) Frequent serial injections of GHRH, GHRP-6, or GHRH analog 585 revealed that intermittent GH responsiveness applies to all the GH secretagogues tested and is a feature of the intact conscious animal. (2) Constant GHRP-6 exposure removed the variability of the GHRH GH responsiveness in the rat and thus speculated that GHRP-6 or GHRH analog injection may synchronize the neuro-mechanism activity that generates spontaneous GH secretion. (3) Whether chronic GHS administration has a trophic action on pituitary somatotrophs needs to be determined because this may be of more clinical value than GHS potency. (4) At maximum dosages in vitro GHRP releases less GH than in vitro GHRH but the reverse occurs in vivo. This suggests that a third factor may be involved in the in vivo GH release by GHRP. (5) Studies in the guinea pig indicate that a centrally administered small amount of GHS has a specific and sensitive central action that is not shared by GHRH. This could support the release of a third factor by GHSs as well as GHRPs. (6) Since SRIF inhibits GH responses and also is a functional SRIF antagonist on GHRP-induced GH release, it is possible that GHRP may functionally inhibit the hypothalamic action of SRIF.

Also Fairhall and Robinson considered that GHRP may be, at times, a hypothalamic SRIF antagonist. This antagonism especially needs consideration during combined effects of GHRP and GHRH on the hypothalamus and/or pituitary sites of action. The studies of Dixon et al. indicate that GHRP-6 directly increases hypothalamic cfos and studies of Okada et al. indicate an enhanced action on food intake with icv GHRP + GHRH.

Second-Generation GHS Chemistry

As published by Art Patchett and colleagues at Merck, the chemical development of MK-0677 initially evolved from our 1975 first DTrp^{2}Met enkephalin analog that specifically released GH in vitro as well as the structure of GHRP-6 which released GH in vivo [31, 32]. The primary objective of Merck was to develop a nonpeptide GHS with high oral activity which indeed was accomplished since MK-0677 has 60% oral availability while that of GHRP-6 was less than 1%. This was effectively accomplished by utilizing the Evans et al. medicinal chemistry structure approach designated "privileged structures" in the GHS design process [52]. By this approach the initial rigid chemical structures of the GHSs were converted to the molecule MK-0677 which had a greater receptor flexibility, adaptability, and affinity. "Privileged structures" refer to molecular units that have appeared as recurring chemical molecules, core structures in a number of different drugs which act on CNS G protein receptors. As discussed in the 1999 chapter on "GHRPs" in the

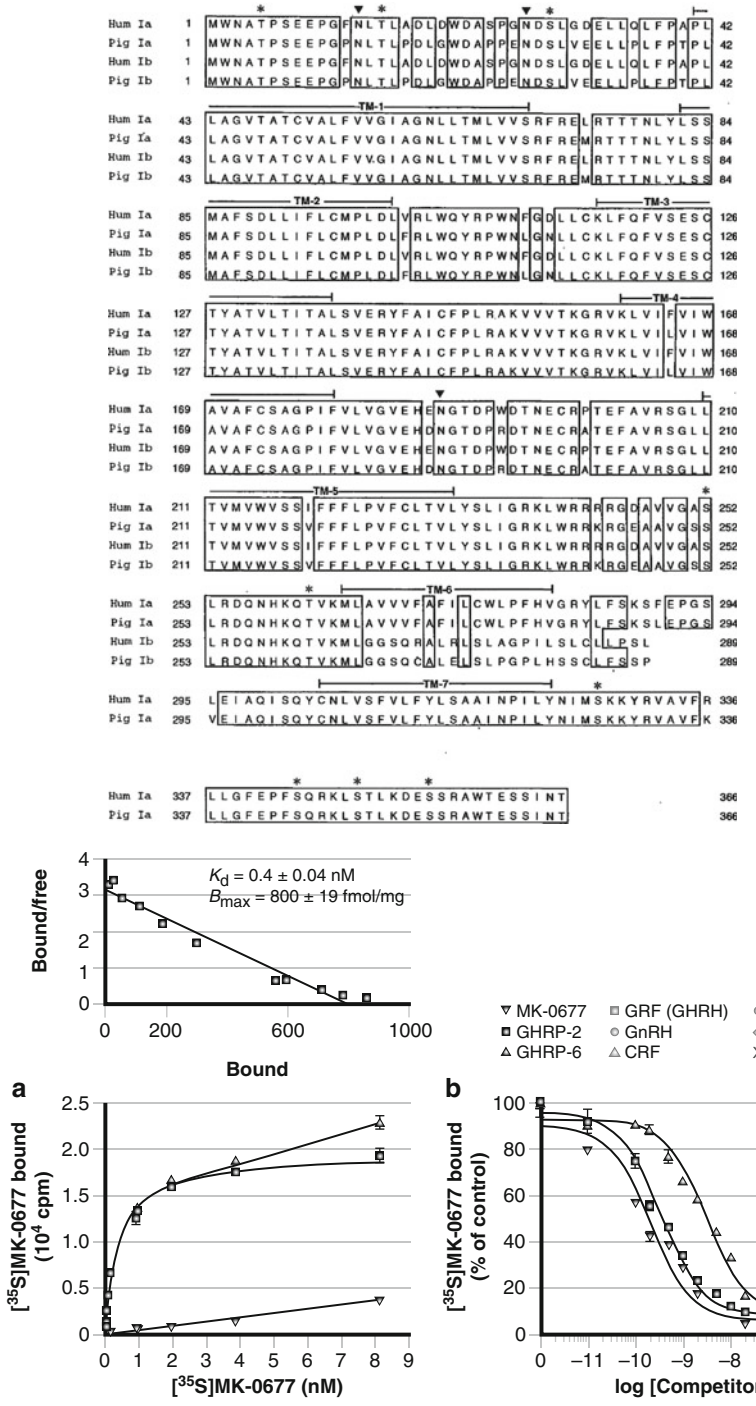
Handbook of Physiology**,** a number of some but not all peptide and nonpeptide GHRPs/GHSs were under development by other groups which had specific and high GH-releasing activity [53].

Although several of the companies pursued development of the GHSs, Merck's program was more in-depth at the basic and clinical levels. The latter resulted in very valuable information of the intracellular signaling transduction system and, in particular, the notable accomplishment of the cloning, expression, and sequence of the GHS receptor designated GHS-R 1a in 1995 and 1996. The Merck group indeed was a true believer in this new GH regulating system and the establishment of the milestone, the GHS receptor, convincingly again forecasts a natural hormone in need of isolation and identification.

GHRP/GHS Natural Receptor

Equally exciting and in particular of fundamental biological importance was the accomplishment of the identification and cloning of the G-protein GHS receptor designated GHS-R 1a by Howard et al. at Merck [48]. Thus after 20 years this became step two in the milestones of the "unnatural to natural" story. This second GHRP/GHS milestone was born by Howard, Smith, Feighner, and Van der Ploeg et al. from the Merck group in 1996 (Fig. 1.3). This 7 transmembrane (TM) G-protein-coupled receptor with high amino acid identity (93%+) was present in human, swine, and rat. By dendrogram amino acid analysis of G protein-coupled receptors GHS-R 1a existed in a relatively isolated position [54]. Closely related family members were hNeurotensin-R1 and TRH-R, i.e., ~35–29%, respectively. Interesting was the chemical identity centered within the TM regions of rhodopsin—family I. Based on the predicted conformational structures, several amino acid residues in TM 3,5,6 appeared to be involved in ligand binding. Also of special note was the identity centered within the TM regions of the rhodopsin—family I group and the GHS-R 1a. Indeed a large amount of valuable basic information evolved via the accomplishment of the isolation and identification of the GHS-R 1a including the intracellular signaling transduction pathway system by Smith et al. [55].

Lex van der Ploeg and Scott Feighner related many of the unique and complicated strategies and technical aspects that lead to the glorious cloning of the GHS-receptor (R) 1a [56, 57]. By utilizing Merck's GHS MK-0677, specific receptors in the hypothalamus and anterior pituitary were demonstrated and a new pathway of GH regulation across vertebrate species (chicken, mouse, rat, guinea pig, horse, cattle, sheep, pig, and humans) was established. These results strongly supported the speculation that the GHSs mimic the effect of a naturally occurring hormone and also were involved in the regulation of pulsatile GH secretion. GHRH, SRIF, and MK-0677 receptors were proposed to induce more than just changes in hormone levels. This concerned effects on the relative concentration of the GHRH, SRIF, and MK-0677 receptors and the kinetics of activation, inactivation, and reactivation of each receptor. Presumably these effects regulated feedback loops and


Hum Ia
Pig Ia
Hum Ib
Pig Ib
TM-1
TM-2
TM-3
TM-4
TM-5
TM-6
TM-7
Bound/free
K_d = 0.4 ± 0.04 nM
B_{max} = 800 ± 19 fmol/mg
Bound
MK-0677
GHRP-2
GHRP-6
GRF (GHRH)
GnRH
CRF
TRH
Galanin
Neuromedin B
a
[35S]MK-0677 bound (10^4 cpm)
[35S]MK-0677 (nM)
b
[35S]MK-0677 bound (% of control)
log [Competitor (M)]

consequently induced GH pulsatile secretion. Since GH oscillations could be initiated and modified by the MK-0677 class of GH secretagogues, the putative natural hormone was speculated to modulate oscillatory mechanisms involved in regulation of GH secretion by triggering and maintaining symmetry breaking**.** In contrast, since IGF-1 was not released episodically, it probably did not affect the symmetry breaking. IGF-1 was postulated to dampen the GH axis by inducing a new set point for the amplitude of the oscillations [55].

In addition, via the GH deconvolution analysis approach the time intervals between pulses occur as a result of the hypothalamic action of MK-0677 which enhances the amplitude and/or frequency of GHRH release. The increase in GHRH pulse amplitude/frequency induced by MK-0677 in turn attenuates the SRIF effect on GH release as discussed by Smith et al. [55].

By 2002, Gnananpavan et al. published data on the distribution of the human bioactive ghrelin GHS-R 1a mRNA expression [58].

GHRP/GHS Receptor Binding and Actions

To add to the reality and excitement of the combined GHRP/GHS story are the results of Feighner et al. [57]. In 1998 they published the in vitro binding results of the GHRP/GHSs before and after single amino acid mutations of the hGHS-R 1a in transmembrane 2,3,5,6. Recorded in Fig. 1.4 is a three-dimensional docking model of the nonpeptide benzolactam spiropiperidine GHSs and the hexapeptide GHRP-6. Mutation of the glutamic acid residue at position 124 in TM 3 resulted in a nonfunctional receptor for each of the three chemical types of GHRP/GHSs. Since each GHRP/GHS has an essential positively charged nitrogen atom at the N-terminus the nonfunctional receptor binding was explained by eliminating the counterion interaction between these three GHSs and the receptor. The TM 2,5,6 mutations resulted in different effects on the binding activity of these three chemically different GHSs**.** By utilization of the single amino acid mutation receptor approach, overlapping binding sites of GHRP-6 and MK-0677 were demonstrated. Based on the predicted conformational structure of GHRP-6 several amino acid residues of TM 3,5,6 were involved in the binding of GHRP-6 and also the nonpeptide GHS MK-0677 as recorded in Fig. 1.4. The interesting speculation was

Fig. 1.3 GHS-R predicted amino acid sequence (*top*). Type 1a and type1b predicted sequences from swine and human GHS-R. Identical residues are boxed. Conserved cysteine residues and the GPC-R signature sequence GluArgTyr^{142} are shaded *gray* in original article. Potential sites for N-linked glycosylation (*arrows*) and phosphorylation (*asterisks*) are shown. TM represents the transmembrane domains. Binding of [^{35}S]-MK-0677 to crude membranes from COS-7 cells transfected with human type 1a GHS-R cDNA (*bottom*). (**a**) Saturation isotherm and Scatchard analysis (*inset*) of [^{35}S]-MK-0677 binding (bound units are femtomole per milligram of protein). (**b**) Competition analysis (0.24 nM [^{35}S]-MK-0677). Reproduced with permission from [48]

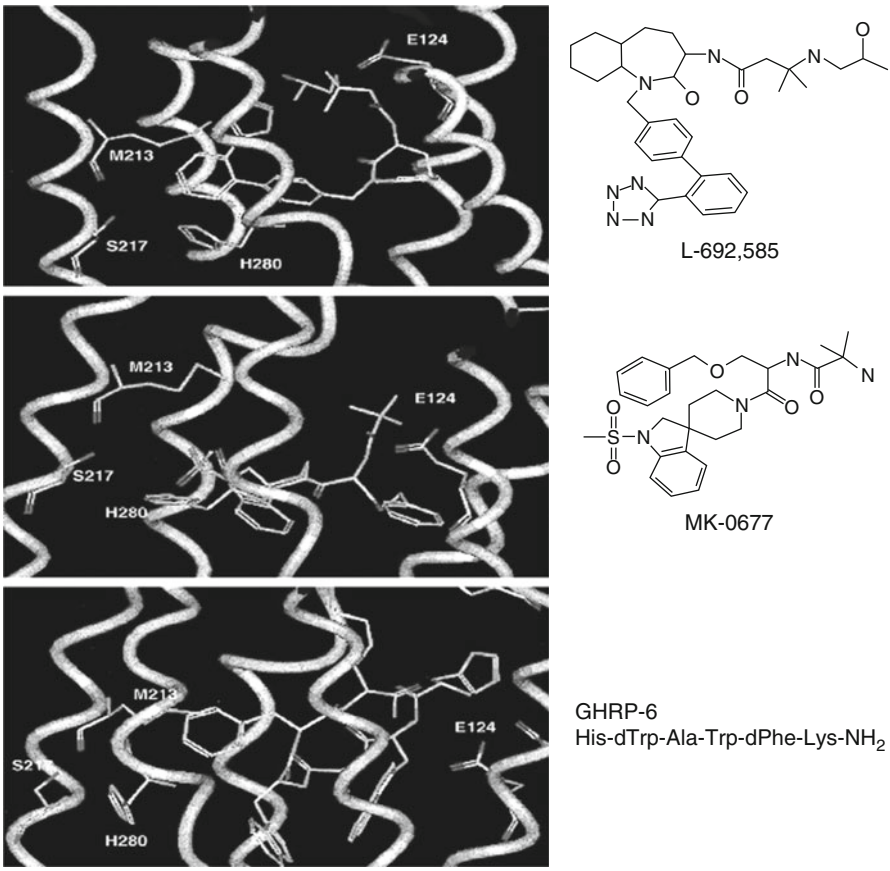


Fig. 1.4 Three-dimensional structure of ligand-binding pocket of the human GHS-R with MK-0677, L-692,585, and GHRP-6 docked. Reproduced with permission from [57]

proposed that the three GHSs probably bind to the same receptor site but by different orientations which may selectively affect the affinity, dissociation constant, potency, and even specificity of the particular secretagogue. The details of these elegant historical accomplishments are published in Endocrine Reviews (1997) by Roy Smith et al. entitled "Peptidomimetic Regulation of GH Secretion" [55].

Previous exciting and unique special receptor results of GHRP began from the initial results of Brigitte Holst and Theodore Schwartz et al. published in 2003 [59]. As recorded in Fig. 1.5, results indicate that the basal activity of the GHS-R 1a when unoccupied by a receptor inverse agonist is 50% above its lowest basal activity indicating that the GHS-R 1a has high constitutive activity. In this same Fig. 1.5, the Sub-P analog, [DArg^{1}DPhe^{5}DTrp^{7,9}Leu^{11}]-Sub-P, which we had found to inhibit the GH response of GHRPs/GHSs and eventually ghrelin both in vitro and in vivo, was reported by Holst et al. to inhibit at low dosages (5 nM) the high basal activity of the unstimulated GHS-R 1a and also to inhibit ghrelin-stimulated

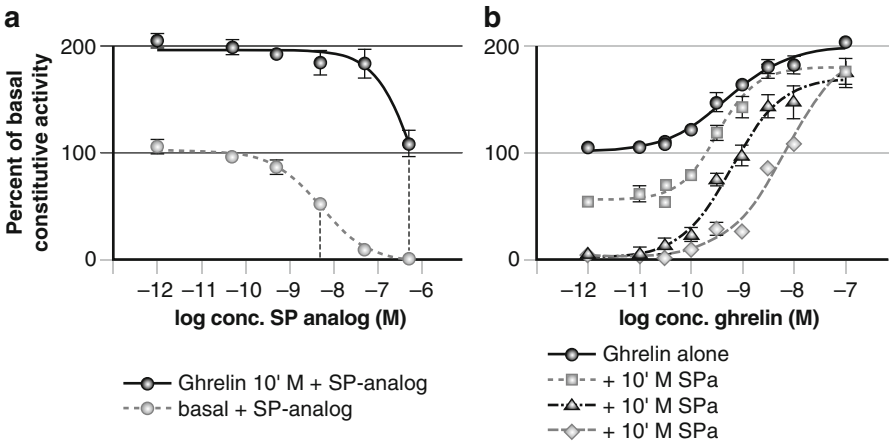


Fig. 1.5 Effect of [DArg[1]DPhe[5]DTrp[7,9]Leu[11]]-Substance P (analog) on the constitutive activity (*full circle*) and in the ghrelin-stimulated IP turnover (*open circle*). (**a**) The IC_{50} for the Substance P analog acting as an antagonist against ghrelin-stimulated signaling was 630 ± 20 nM, whereas its IC_{50} as an inverse agonist was 5.2 ± 0.7 nM. (**b**) Dose–response curves for ghrelin in the presence and absence of Substance P analog in three different concentrations: 10^{-6} M (*diamonds*), 10^{-7} M (*triangles*), and 10^{-8} M (*squares*). Reproduced with permission from [59]

activity of this receptor but only at high dosages (630 nM) [27–29]. Thus, this Sub-P analog has both inverse agonist and competitive GHRP/GHS receptor antagonist activity. Also, recently we have synthesized ghrelin-derived molecular receptor antagonists [60]. Whether this or related analogs of this chemical type have inverse activity is currently unknown.

The results of Holst et al. and Pantel et al./Wang et al. recorded in Fig. 1.6a, b support that the time-on constitutive activity of the ghrelin receptor and the genetic phenotype of short stature and/or obesity meaningfully come together [61]. We previously presented and discussed these results of Holst et al. in relationship to the important functional genetic mutation of the GHS-R 1a (ghrelin-R), low-dose icv infusion of [DArg[1]DPhe[5]DTrp[7,9]Leu[11]]-Sub P to rats to assess in vivo effects as an inverse agonist, and, in addition, speculation of Zigman et al. that high constitutive ghrelin-R activity may occur at CNS sites that do not have ready access to circulating ghrelin [62–64].

Marta Korbonitz in 2002 summarized more comprehensive, holistic approaches to better understand the development of pituitary tumors. The possible roles of the hexarelin GHRP and pituitary receptors were determined [65].

Physiological and histochemical findings in rats in 2001 support that an additional neuroendocrine pathway may exist to regulate pulsatile GH secretion possibly through the influence of the newly discovered GHS natural peptide, ghrelin [66]. The speculation that ghrelin may serve as a signal to link energy metabolism and GH secretion was demonstrated by Tannenbaum et al. because administration of a GHRH antiserum inhibited the ghrelin-induced GH response [67].

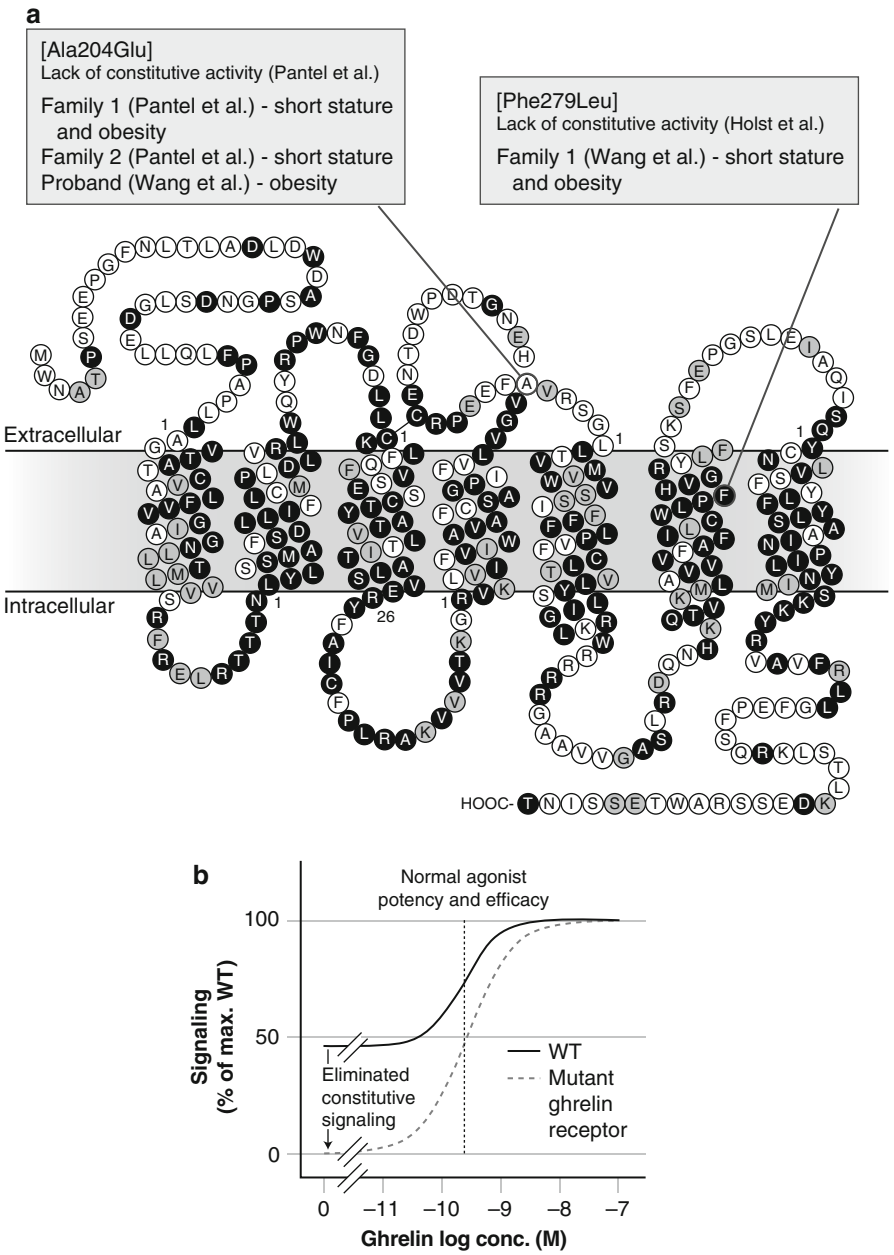


Fig. 1.6 Natural mutations selectively eliminating the constitutive signaling of the ghrelin receptor that are associated with short stature and possible obesity. (**a**) Serpentine model of the human ghrelin receptor and its closest homologue, the motilin receptor, is indicated. (**b**) Schematic diagram of the effects of these natural mutations in eliminating the constitutive signaling of the ghrelin receptor (*arrow* to the *left*) without affecting the potency or efficacy of the natural ligand, ghrelin (*dotted vertical line* corresponding to the EC_{50}). Reproduced with permission from [61]

Clinical

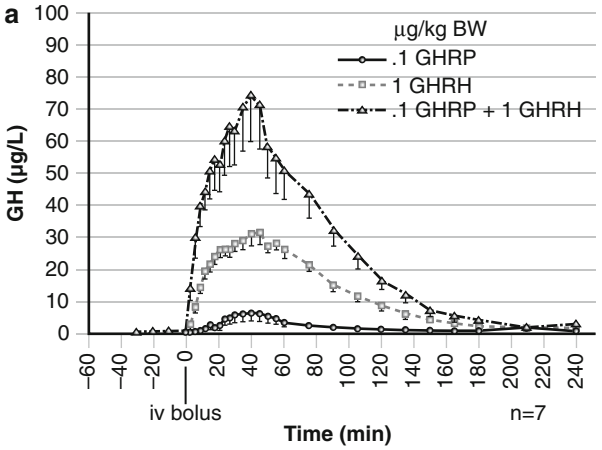
The 1990–2000 GHRP/GHS historical saga from a clinical viewpoint only emphasizes its complexities and incompleteness especially in regard to how the subject continues to evolve. The historical beginning started with GH release and synergistic GH release by combined GHRP-6 and GHRH administration [68]. Additionally, synergistic GH release was considered of physiological importance because this requires the integrated actions of GHRP-6, GHRH, and SRIF. Eventually it was demonstrated that ghrelin, GHRH, and SRIF induced these same synergistic effects on GH secretion in both animals and humans.

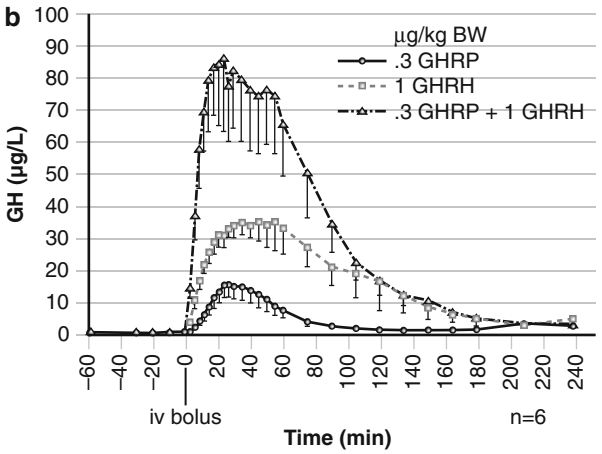
Between 1989 and 1990 the first demonstration of GHRP-6 activity on GH release in humans was demonstrated as well as its unique GH actions in combination with GHRH. Ilson et al. of Smith Kline and French, utilizing an SK&F preparation of GHRP-6, selectively stimulated GH release but not the release of other hormones in a dose–response study in normal men following 30-min iv infusions [69]. Serum LH, FSH, TSH, and ACTH were not affected by GHRP-6. These results agreed with their previous in vitro results and also their in vivo results in a variety of animal species.

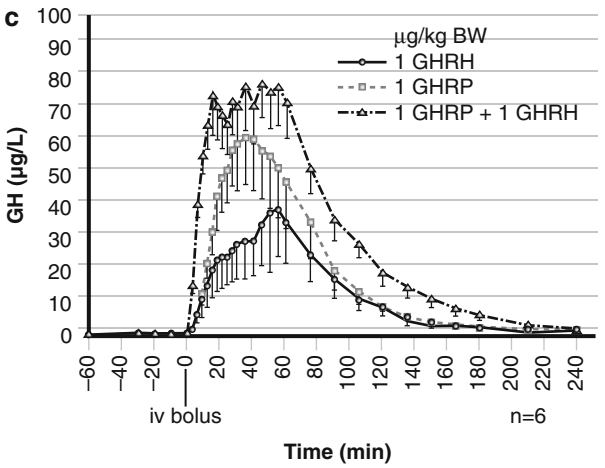
In 1990 studies of Bowers and Thorner et al. published that GHRP-6 was a potent GH secretagogue in normal men [68]. The iv bolus of 0.1, 0.3, and 1 μg/kg of GHRP-6 increasingly elevated GH release (Fig. 1.7). As in the studies by Ilson et al. in normal men, GHRP-6 markedly released GH without increasing TSH, LH, or FSH but did raise PRL and cortisol levels <25% but these levels remained within the normal range. When the above three GHRP-6 dosages were administered with a constant 1 μg/kg dose of GHRH by iv bolus there was a marked synergistic dose–response-related release of GH at the dosages of 0.1 and 0.3 μg/kg of GHRP-6 and an additive GH release by 1 μg/kg of the two peptides concomitantly. Thus the novel unique synergistic GH release of combined GHRH and GHRP-6 forecasts that this probably is a fundamental physiological action that would be expected of GHRH and the putative natural GHRP hormone. Besides in humans, this dose-related synergism was equally dramatic in an array of animals and thus the cellular and molecular mechanisms involved would be of prime physiologic and pathophysiologic importance to reveal and understand.

Craig Jaffe and Ariel Barkan et al. as well as Huhn and Thorner et al. reported in 1993 on the continuous infusion of saline or GHRP-6 for 36 or 24 h in normal young men [70, 71]. In both studies GHRP-6 increased the amplitude of the normal spontaneous GH pulses without increasing the frequency of secretion. Also in the Jaffe study iv bolus TRH, GHRH, and GHRP-6 were administered at the end of the infusion period but still during the continuous infusion of GHRP-6. TRH did not release GH while the GH response to GHRH was increased during the first two but not the last GHRH pulse and the GHRP-6 GH response was decreased [70]. These results are recorded in Fig. 1.8.

In 1996, Chapman and Thorner et al. published the first orally potent GHS MK-0677 clinical study in healthy older men and women. It was concluded from


a
µg/kg BW
.1 GHRP
1 GHRH
.1 GHRP + 1 GHRH
GH (µg/L)
iv bolus
n=7
Time (min)


b
µg/kg BW
.3 GHRP
1 GHRH
.3 GHRP + 1 GHRH
GH (µg/L)
iv bolus
n=6
Time (min)


c
µg/kg BW
1 GHRH
1 GHRP
1 GHRP + 1 GHRH
GH (µg/L)
iv bolus
n=6
Time (min)

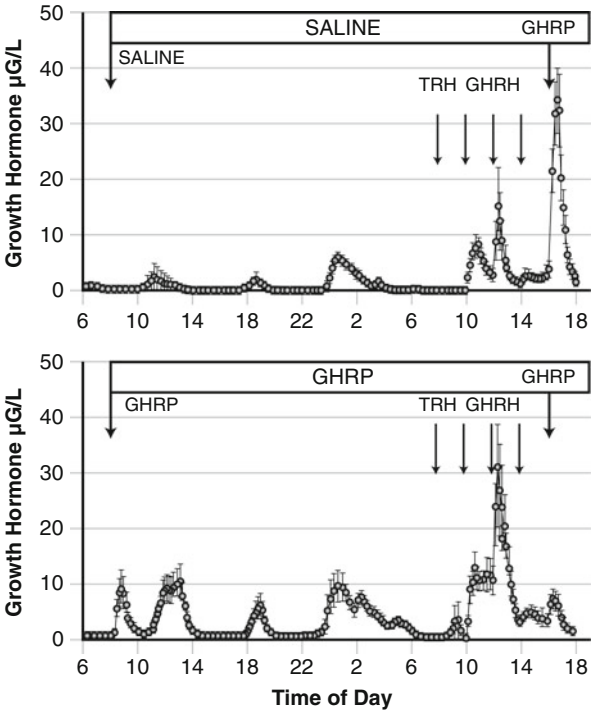


Fig. 1.8 Effect of prolonged GHRP infusion on pulsatile GH in normal men. Mean (±) SE plasma GH concentrations in nine men during saline (*upper panel*) and GHRP (*lower panel*) infusions from 0600 h on day 1 to 1800 h on day 2. Reproduced with permission from [70]

the study that MK-0677 could be as effective in releasing GH in older subjects as GHRH is in younger subjects. They projected if GH responses can be maintained during chronic MK-0677 therapy, GH and IGF-1 levels in older and younger subjects could be the same [72]. Also, the effects of MK-0677 obtained by Svensson et al. in 1998 on fat free and fat mass in obese subjects have been summarized and reported [62].

In 1998, Pandya and Barkan et al. demonstrated that in normal young men a GHRH antagonist substantially but not completely inhibited the GH release of GHRP-6 [73]. Additionally, both GHRH and GHRPs have definite relationships to acromegaly. GHRH induces acromegaly in patients with functional secreting pancreatic as well as lung tumors. GHRP-6, like GHRH, acts directly on the pituitary somatotroph cells to release GH and again, like GHRH, via complementary but yet

Fig. 1.7 GHRP plus GHRH synergism. Each subject received both peptides administered separately and in combination. Note that synergism was most evident at 0.1 µ(micro)g/kg GHRP (**a**) when the simultaneous delivery of GHRP and GHRH produced a greater GH response than the sum of the responses with either peptide administered separately. When the doses of GHRP were increased to 0.3 and 1.0 µg/kg (**b** and **c**), this synergism became progressively less evident. Reproduced with permission from [68]

different intracellular signal transduction pathways. Alster and Barkan et al. revealed that acute iv bolus GHRP-6 markedly increases GH release in acromegalic patients even more frequently and in amounts greater than GHRH but tantamount to TRH [74]. Thus even under pathophysiological conditions the existence of a natural GHRP hormone and receptor again was supported.

In 1996, 1997, and 1998 clinical studies in short stature children with various degrees of GH deficiency were performed by three different groups, i.e., Laron et al., Pihoker et al., Mericq, Cassorla, and Merriam et al., by chronically administering hexarelin or GHRP-2 intranasally or subcutaneously for 6 months to 2 years. The effects on body height were definitely increased in all three studies but were suboptimal probably due to the method of peptide delivery [53, 62]. In addition, these same three groups and also Tuilpakov et al. published diagnostic studies in children with varying degrees of GH deficiency using GHRP-1/GHRP-2 [53, 62].

In our 30-day studies with GHRP-2 and GHRP-2 + GHRH pulsatile secretion of GH and sustained enhanced IGF-I levels underscore the complementary physiological action of these two peptides [75]. Under combined clamped administration of GHRP-2 + GHRH, the feedforward prolonged stimulation of normal pulsatile secretion of GH is maintained and augmented and IGF-I levels are raised to within normal range over the entire 30-day infusion period. The results again underscore GHRP-2, GHRH, and SRIF as the primary regulators and GH and IGF-I as secondary regulators of GH pulsatile secretion.

The comparative pulsatile GH levels and area under the curve (AUC) as well as serum IGF-I levels during continuous 24-h sc infusion of placebo, GHRH, Ghrelin, GHRP-2, and GHRP-3 of a 68-year-old obese woman are recorded in Fig. 1.9. GHRP-3 is a newly developed, chemically and biologically stable, highly potent GHS that is 3–5 times more potent than GHRP-2 [62]. The high potency and efficacy of GHRP-3 in activating the GH-IGF-I axis demonstrate that it can be readily developed for incorporation into a 3–6-month depot formulation for clinical therapy with the advantage of having low exposure to the subject/patient.

Because of the page limitation and the large number of 1990–2000 clinical studies on the acute and chronic effects of peptide and nonpeptide GHRPs/GHSs on GH secretion in humans, six general reviews have been selected in order to convey a broader clinical historical overview of this aspect of the subject [53, 55, 76–79]. Additionally, the review by Giustina and Veldhuis was included because it emphasized and incorporated relevant actions of GHRH and SRIF together with the integrated effects of the thyroid, adrenal, and gonadal hormones as well as other relevant factors which significantly influence the GHS actions not only on GH secretion but also on select other nutritional, metabolic, gastrointestinal, and CNS actions [80]. The latter studies and results have not been included in this present 1974–2000 historical perspective.

The above-listed reviews include historical clinical GHRP/GHS achievements between 1990 and 2000 on all ages and both sexes. Also, they reveal new aspects of physiological and pathophysiological states, diagnostic tests, short- and long-term therapy with different GHRPs and GHSs by different routes of administration, acute and chronic critical illnesses, under- and overnutrition, and specific endocrine states. The latter has been extended to include multiple metabolic and GI states but these

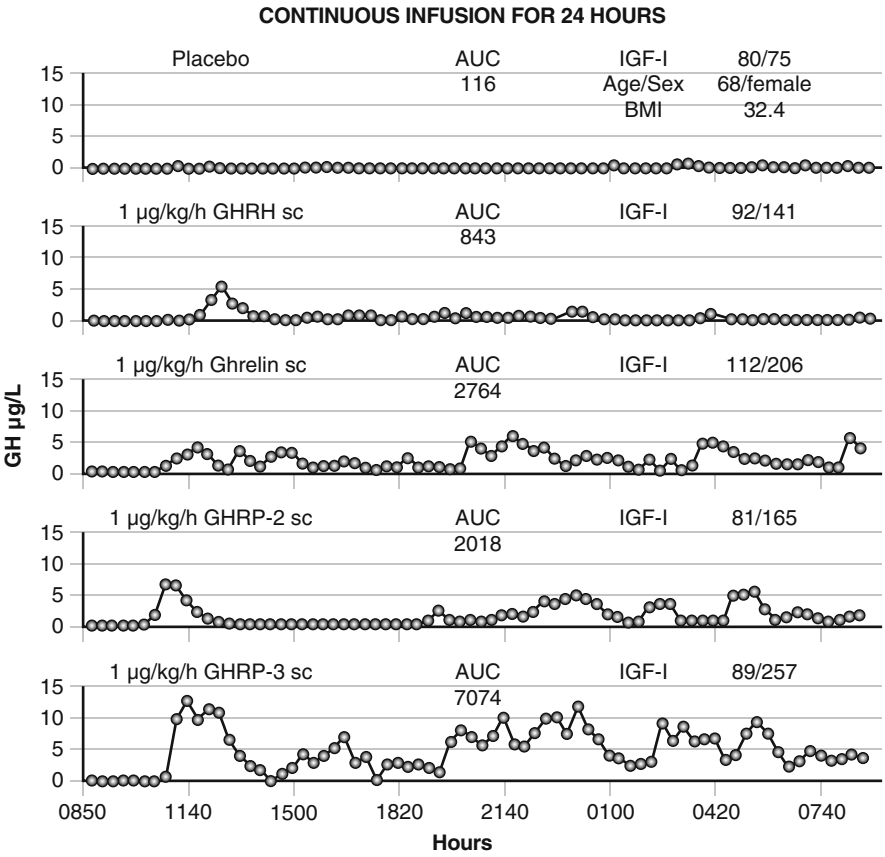


Fig. 1.9 Continuous 24-h sc infusion of Placebo, GHRH, Ghrelin, GHRP-2, and GHRP-3 in the same obese subject. Reproduced with permission from [62]

were not included in the present brief historical perspective. These topics will be discussed by other authors in this book specifically in reference to ghrelin. Besides direct basic and clinical early studies, the contributions of Berry Bercu and Richard Walker merit special recognition as editors of the series of Symposia on the GHRP–GHS evolution.

Isolation of Ghrelin

Preliminary evidence was obtained in earlier studies which supported the presence of a natural GHRP hormone in the hypothalamus. As recorded in Fig. 1.10, partially purified fractions from porcine hypothalami indicated the existence of the putative natural GHRP hormone [76].

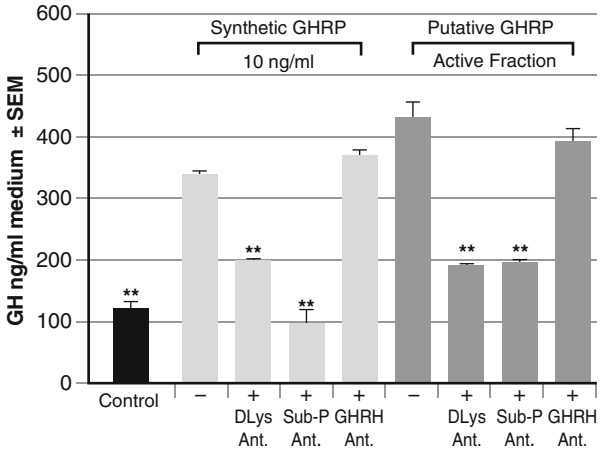


Fig. 1.10 Isolation of partially purified putative natural GHRP from porcine hypothalami assayed in rat pituitary dispersed cell culture. These are results of a purified fraction that are considered to represent the activity of the putative natural endogenous GHRP. GH was released by both the unnatural synthetic GHRP and the putative natural GHRP alone as well as with the GHRH antagonist. Both DLys^{3}-GHRP-6 and Sub P antagonist ([DArg^{1}DPhe^{5}DTrp^{7,9}Leu^{11}]-Substance-P) inhibited the GH release induced by the synthetic and the putative natural GHRP. The fraction did not increase cAMP release. Dose of antagonist is 10 ng/ml. ***p* value is <0.01 to <0.001. Reproduced with permission from [76]

A finale that many waited patiently for as long as 25 years was the 1999 publication of the isolation and identification of the natural GHRP/GHS hormone, ghrelin, which is a 28 amino acid peptide with an *n*-octanoate molecule covalently linked via an ester bond to the Ser^{3}-OH moiety by Kojima and Kangawa et al. in Osaka, Japan [81]. Obviously this exotic molecule isolated from the stomach "speaks" for itself and is multidimensional at many different levels including the stomach. In addition, it is a major regulator of GH secretion and food intake in animals and humans and has an extended evolutionary presence. In addition, ghrelin has extended pleiotrophic, metabolic actions. As recorded in Fig. 1.11, the isolation and structural identification were first published in Nature on December 9, 1999. The GHS/ghrelin receptor and natural hormone will continue to be an everlasting shining star scientific achievement [82].

In 2001, Gurd and Tannenbaum et al. published the following on the interrelationships between ghrelin/SRIF/GHRH and the regulation of pulsatile GH secretion: (1) ghrelin administered either centrally or peripherally exerts potent, time-dependent GH-releasing activity under physiological conditions; (2) ghrelin does not release GH by inhibiting endogenous SRIF release; (3) SRIF is a functional antagonist of ghrelin acting centrally as well as at the level of the pituitary gland; and (4) the GH response to ghrelin requires an intact endogenous GHRH system [83]. Additionally, in 2002 Tannenbaum et al. published findings in rats which supported that ghrelin may be a critical hormonal signal of nutritional status to the GH neuroendocrine axis serving to integrate energy balance and the growth process [84].

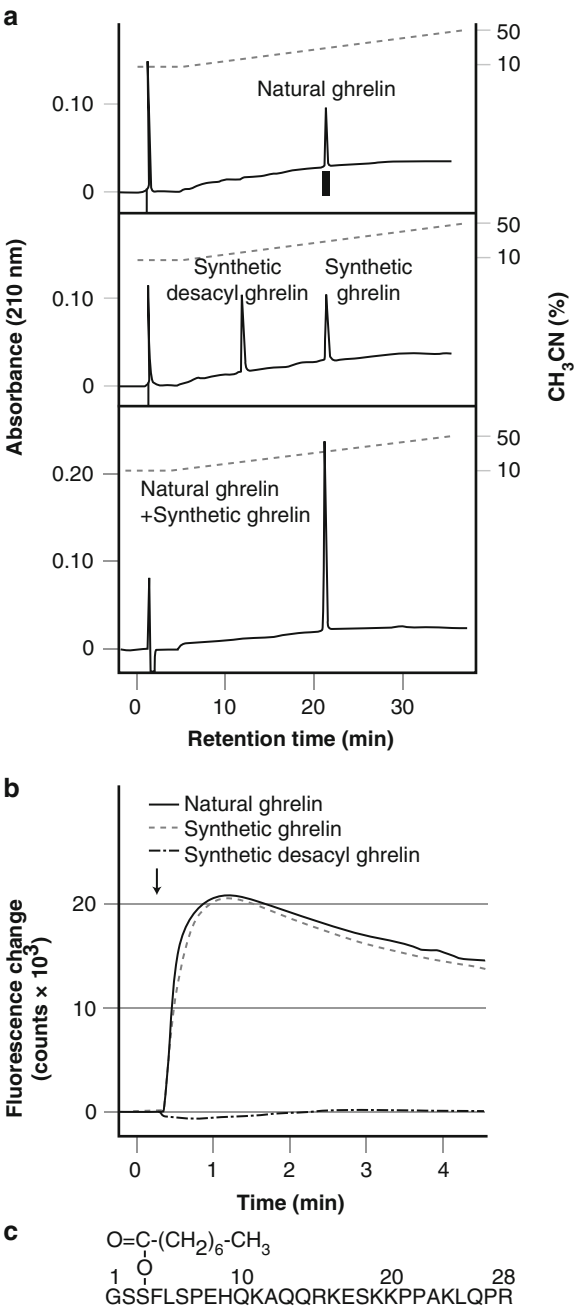


Fig. 1.11 Identification of the *n*-octanoyl modification in ghrelin. (**a**) Chromatographic comparison on RP-HPLC of natural ghrelin (*top panel*), synthetic ghrelin, and desacyl ghrelin (*middle panel*), and co-migration of natural and synthetic ghrelin (*bottom panel*). (**b**) Time courses for changes in $[Ca^{2+}]_i$ in CHO-GHSR62 cells induced by natural ghrelin, synthetic ghrelin, and desacyl ghrelin. Each peptide (10^{-8} M) was added at the time indicated by the *arrow*. (**c**) Structure of rat ghrelin. Reproduced with permission from [81]

Hataya et al. found that ghrelin and GHRH, like GHRP-2 and GHRH, is equally synergistic [85]. In our limited initial comparative GHRP-2 and ghrelin studies, both alone and together with GHRH by continuous sc infusion for 24 h, the GH and

IGF-I effects were essentially the same [86]. Both peptides increased the normal pulsatile GH secretion and, like GHRP-2, ghrelin administered together with GHRH by iv bolus released GH synergistically.

Each year since 1990 new unique, novel, nonlinear actions, dimensions, unexpected clinical actions/effects of GHRPs/GHSs, and finally ghrelin are still being revealed. These results are both surprising and non-surprising but also a FINALE is not readily apparent. Because of page limitation, we have selected a series of comprehensive review publications to convey most but not all of the 2000 preghrelin clinical results. Obviously unnatural GHRP/GHS clinical results only obtusely and incompletely forecast the complete clinical results of natural ghrelin which itself is unique and probably even more exciting than realized presently. A special dimension of both potential major basic and clinical importance not included is the early seminal ghrelin findings of Date et al. which impart that stomach ghrelin CNS effects are mediated via both the gastric nerve and the peripheral circulation [87].

Various different types of in vitro binding receptor techniques and approaches have been utilized that will not be discussed as part of this ghrelin history. These studies have increased in frequency and type particularly in regard to in vitro and in vivo functional effects rather than binding indices of the response**.** So far it has been difficult to definitely evaluate when the results obtained reflect technical assay issues, receptors yet to be established, low receptor concentration, and select paracrine/autocrine function under pathophysiological/pathological conditions such as tumors, inflammation, metabolic disorders, etc. A salient current esoteric example also not discussed is the projected biological activity of the desacyl ghrelin molecule. So far, the receptor for the desacyl ghrelin molecule is yet to be identified.

Regulation of GH Secretion by Hypothalamic Peptides [78, 80, 88, 89]

1. Three-peptide concept of regulation (Fig. 1.12)
   GH secretion is controlled by an ensemble of interlinked peptide, viz, GHRH, SRIF, and ghrelin (GHRP) [78, 80, 88, 89]. Clinical studies, laboratory experiments, rare sporadic mutations, targeted gene silencing, and biomathematical models establish that all three signals regulate GH secretion (Fig. 1.12). A clarion implication of the concept of integrative control is that no one peptidyl effector either operated alone or can be understood adequately when studied alone. A major unanswered question is how factors like age, sex steroids, and body composition modulate the core regulatory ensemble, thereby altering GH and IGF-1 availability. Salient investigational challenges in prosecuting this theme are strong interdependence among age, estradiol availability and abdominal visceral fat mass, and dearth of reliable experimental strategies and validated analytical tools to probe interlinked roles of GHRH, SRIF, and ghrelin.

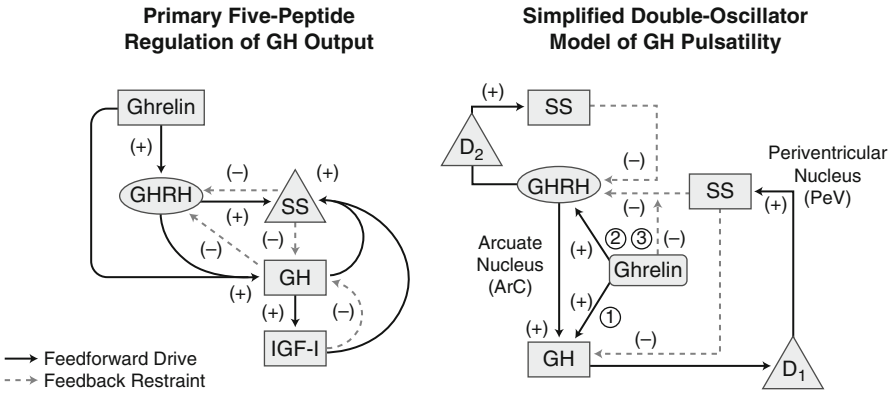


Fig. 1.12 Ensemble GH axis viewed in relation to five key peptide signals and their minimal interactions (*left diagram*). Formalized analytical construct of SS-directed self-sustaining oscillations. D_1 and D_2 are time delays. Note topographic and functional separation of PeV and ArC SS neurons. Ghrelin is integrated into this model at three levels (*right diagram*)

(a) Role of SRIF

SRIF was the first major GH-regulating brain peptide discovered. SRIF controls the size and the timing of GH pulses via (a) direct pituitary effects and (b) intrahypothalamic actions. At the pituitary, SRIF blocks the exocytosis but not the synthesis of GH molecules and resensitizes somatotrope GHRH receptors via phosphatase activation. GHRH stimulates GH biosynthesis, thereby augmenting GH stores even when SRIF is present. In contrast, ghrelin does not drive GH gene expression in the adult. Both GHRH and ghrelin induce GH exocytosis when noncompetitive inhibition by SRIF is relieved. Thus, intermittent exposure of somatotropes to SRIF serves to expand and release GH stores under joint stimulation by GHRH and ghrelin.

In the hypothalamus, SRIF inhibits GHRH neurons directly via synaptic connections. Transsynaptic inhibition blocks both the secretion of GHRH into pituitary portal blood and the firing of GHRH neurons. Therefore, cyclic neuronal SRIF outflow governs not only the amount but also the timing of GHRH and thereby GH secretory burst. Ghrelin, an endogenous GHRP, induces GHRH secretion by arcuate-nucleus neurons and antagonizes the neuronal effects of SRIF, including its repression of GHRH release. In contrast, ghrelin does not diminish the secretion of SRIF into portal blood.

Peripherally injected SRIF acts directly on the pituitary gland, where it blocks exocytosis of GH molecules. Blood-borne SRIF does not enter the brain because infusion of SRIF directly into cerebral ventricles actually stimulates GH secretion, putatively by successively inhibiting and disinhibiting arcuate GHRH neurons**.** In fact, systemic injection of octreotide, a brain-permanent agonist of SRIF receptors, initially inhibits and then (as the drug decays) disinhibits GHRH

secretion into portal blood in the conscious ram. Conversely, repeated administration of a novel linear hexapeptide antagonist of SRIF receptors stunts somatic growth in the male rat, inferably by blocking SRIF's cyclic control of GHRH secretion. Likewise cessation of a peripheral SRIF infusion elicits a rebound-like pulse of GHRH and GH. The infusion lowers systemic GH concentrations, which withdraws GH's feedback drive of SRIF outflow, thereby promoting GHRH release. Although secreted GHRH cannot stimulate GH exocytosis during SRIF infusion, hypothalamic GHRH continues to induce the synthesis of GH stores to evoke GH release. A GH pulse emerges because brain SRIF outflow to the pituitary gland is reduced and brain SRIF is augmented by the low GH feedback state. A prediction is that hypothalamo-pituitary and systemic ghrelin concentrations would activate CNS and pituitary mechanisms to potentiate GHRH stimulation. Thus, the magnitude and timing of GH responses to SRIF-feedback withdrawal confer a unique window into endogenous regulatory mechanisms.

(b) Ghrelin's role

Peripherally infused ghrelin acts upon the hypothalamus and pituitary gland. In the animal hypothalamus, ghrelin stimulates GHRH secretion, antagonizes neuronal inhibition of SRIF, and induces hunger. Hypothalamic effects of ghrelin mediate synergy with GHRH in vivo, because synergy vanishes in patients with hypothalamo-pituitary disconnection and there is no direct synergy on pituitary cells in vitro. Moreover, hypothalamic effects require GHRH release because GHRP-stimulated GH secretion is reduced by >90% in normal adults administered a GHRH-receptor antagonist and in rare patients with inactivating mutations of the GHRH receptor. GHRP does not regulate the basic interneuronal SRIF–GHRH pulse-generating mechanism, inasmuch as continuous systemic infusion of GHRP/ghrelin augments the size but not the frequency of GH pulses.

(c) GHRH's role

Systemically delivered GHRH, like SRIF, probably acts exclusively on the pituitary gland. Accordingly, continuous iv infusion of tumoral secretion of GHRH markedly amplifies the size but not the frequency of GH pulses. Even if GHRH did enter the CNS, frequency encoding of GH pulses seems to arise from reciprocal synaptic interactions between hypothalamic SRIF and GHRH neurons without requiring brain GHRH receptors. This point is verified by the fact that patients with inactivating mutations of the GHRH receptor maintain a normal frequency of GH pulses, albeit at 30-fold lower amplitude.

(d) Autoregulation of GH pulses

The role of peptides in the generation of successive GH pulses can be viewed as follows. When a GH pulse occurs, rising GH concentrations act via negative feedback on CNS GH receptors to trigger periventricular-nucleus release of SRIF. This outflow represses arcuate GHRH neurons via direct synaptic contacts and inhibits pituitary GH release via the portal system. The subsequent fall in systemic GH concentrations removes drive to paraventricular SRIF neurons. Withdrawal of SRIF outflow disinhibits GHRH neurons and GH exocytosis, thus evoking a coordinated pair of GHRH and GH pulses. Available ghrelin amplifies

the size of GHRH and GH pulses via facilitative actions on both arcuate and the pituitary gland and opposition to SRIF in the arcuate. These concepts can be modeled mathematically.

2. Synergistic effects of peptide infusion
   Dose–response analyses establish that ghrelin's stimulation of GH secretion is asymptotic. Attainment of maximal GH secretion denoted emergence of a de facto rate-limiting step in ghrelin receptor–effector response pathways. One cannot determine directly in the human by electrophysiological criteria whether the rate-limiting step reached is maximal enhancement of neuronal GHRH release and/or maximal silencing of SRIF action. However, consider the consensus observation that ghrelin synergizes with a maximal GHRH stimulus in vivo, but not in vitro. In vivo synergy is explicable if ghrelin releases an (unknown) activator and/or opposes the inhibitory actions of available SRIF. The second inference could be tested by simultaneously infusing maximally effective doses of all three, ghrelin, GHRH, and L-arginine. The rationale is that adding L-arginine to the 2-peptide stimulus would increase GH secretion further only if endogenous SRIF restraint were not fully relieved by ghrelin. This clinical experiment has not been done rigorously as yet. Two derivative studies have been reported, wherein ghrelin and L-arginine were infused together without GHRH. Under these conditions, synergy occurs when a submaximal but not a maximally effective ghrelin is used with L-arginine. A provisional inference is that infusion of a maximally effective ghrelin stimulus already enforces maximal SRIF withdrawal, thereby abolishing any additional effect of L-arginine (Fig. 1.12).
3. Aging
   Growth hormone and IGF-1 concentrations decline exponentially with age beginning in young adulthood, thus resulting in progressive biochemical hyposomatotropism. The effect of aging is significant because epidemiological data correlate hyposomatotropism with osteopenia, sarcopenia, intraabdominal adiposity, insulin resistance, hyperlipidemia, increased atherosclerotic risk, and diminished quality of life. From a mechanistic perspective, the fall in systemic GH and IGF-1 availability is due principally to decreased GH secretion. In particular, aging does not alter GH kinetics, reduce hypoglycemia-stimulated GH release, or impair hepatic IGF-1 production in response to exogenous GH. The cause of hyposomatotropism in this setting is not established. However, the three most powerful predictors of relative GH deficiency are age, sex-steroid depletion, and increased AVF.
4. Sex steroids
   Estrogen is the primary sex-steroid agonist of GH secretion in both women and men. Estrogens are synthesized from androgen substrates via the aromatase enzyme expressed in the brain, pituitary gland, ovary, fat, muscle, liver, kidney, and other tissues. The actions of E_2 are multifaceted because they are mediated via at least 3 major signaling pathways, ER alpha, ER beta, and membrane receptors. They are modified by prominent targeted cell-specific factors and diversified

by reciprocal interactions among estradiol, GH, and IGF-1. Given such biological complexity, a compelling investigative need is to dissect how age, estrogen, and visceral adiposity jointly regulate GH secretion.

In relation to age and relative adiposity, a significant recent discovery is that in an experimentally estrogen-replete milieu age contributes to >75% and AVF <25% of the variability in fasting and secretagogue-stimulated GH secretion in post- and premenopausal women. Analogously in an experimentally estrogen-deficient milieu, postmenopausal women maintain 50% lower GH and IGF-1 concentrations than premenopausal individuals. The combined data clearly distinguish a prominent impact of age, independently of estrogen availability and AVF on the GH/IGF-1 axis in healthy women.

5. Species selectivity
   Laboratory and clinical studies indicate that coordinated interactions among systemic, hypothalamic, and pituitary effectors, such as peptides, sex steroids, thyroxine, cortisol, or free fatty acids, govern pulsatile GH secretion. Nonetheless, the species specifically of sex-steroid action makes inferences gained in the rat, mouse, pig, and sheep illustrative of rather than definite to the human. For example, estrogens repress and non-aromatizable androgens increase pulsatile GH secretion in the rat, but exert opposite effects in the human. Thus caution is appropriately applied in extrapolating inferences across species.
6. Ghrelin and GHRH interactions
   The pivotal nature of amplifying interactions between ghrelin peptide and GHRH neurons has been established in murine gene-silencing models and in spontaneous mutations of the human ghrelin-receptor gene. In the transgenic mouse, knockdown of ghrelin-receptor gene expression in brain GHRH (tyrosine hydroxylase-expressing) neurons decreases GH and IGF-1 concentrations and somatic growth by 30% in the female animal. The mechanisms involve a reduction in GH pulse size, with no change in frequency, and a decrease in the density of hypothalamic GHRH-expressing neurons. These data indicate that ghrelin amplifies the mass of GHRH secreted and also is trophic to GHRH neurons. In humans, a missense mutation of the ghrelin-receptor gene that decreased its cell surface expression and reduced constitutive signaling activity in two separate pedigrees presented with short stature. Albeit not yet assessed directly, one would predict that such patients have small GH pulses of normal frequency, akin to the pattern recognized in the murine model of ghrelin-receptor knockdown. Small pulses of normal frequency are predicted if the CNS ghrelin receptor mediated amplification of GHRH outflow without impinging on the interneuronal (transsynaptic) GHRH–SRIF pulse renewal mechanism.

   What are the implications of putatively impaired ghrelin drive in older adults? To explore this query, we performed a 1-month trial of continuous sc GHRP-2 infusion in healthy aging men and women. Evaluation at 30 days demonstrated that GHRP-2 infusion stimulates pulsatile GH secretion by >1.45-fold and elevated IGF-1 concentrations by >1.65-fold. What is not known is whether favorable body-compositional biochemical and functional performance changes

can be induced by such regimens. However, preliminary investigations by Nagaya et al. reported trophic effects of ghrelin (2 μg/kg iv) twice daily for 3 weeks in cachectic patients with congestive heart failure and COPD. Beneficial effects included increases in cardiac ejection fraction, peak oxygen consumption, skeletal muscle strength, and Karnofsky performance score. A compelling new possibility would be continuous combined (two peptides) stimulation of GH secretion in cachectic and catabolic states. The goal would be to limit the negative acute metabolic effects of ghrelin on the enteropancreatic insulin axis by restricting the ghrelin/GHRP dose.

The dimensions and full spectrum of the physiological and pathophysiological roles of the GHRPs, GHSs, and ghrelin still are in an early stage. This brief historical summary focuses on GHRP/GHS/ghrelin regulation of GH secretion at the obvious expense and neglect of the effects of the ghrelin system/network on metabolism and over- and undernutrition, etc. Parenthetically, the major effect may be on metabolism which in turn modulates regulation of GH secretion. The third possibility is that the dual GH and metabolic regulated effects are a new novel hormonal system with multiple functional and variable unique physiological roles.

What has become increasingly apparent from a number of different developed chemical types of GHRPs/GHSs and broad variety of in vitro and in vivo animal/human biological effects is the tremendous convoluted complexity of the subject which still is evolving. Evidence indicates that the primary CNS and/or peripheral biological effects and broad spectrum of possible clinical objectives of GHRPs/GHSs/ghrelin greatly depend on the agent's chemistry, dosage, route of administration, and clinical disorder as well as whether the therapeutic approach is to induce physiological or phrmacological effects.

It seems appropriate to end this still evolving subject of ghrelin with the following quote. When Pablo Picasso was asked for his opinion at a "centennial art exhibition" he adroitly and quickly sketched the alphabet letters in random order, shape, size, and color and said "you put them together."

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Chapter 2
Enzymatic Processing of Ghrelin Precursor

Masayasu Kojima and Kenji Kangawa

Abstract Ghrelin, an orexigenic peptide hormone from stomach, generally contains an acyl modification at the third serine residue. The serine is modified with a medium-chain fatty acid, typically *n*-octanoic acid. Importantly, this modification is essential for the biological activity of ghrelin. Conservation of the sequence and the required acyl modification at the third residue suggest that ghrelin undergoes a precise series of processing steps. The enzyme that catalyzes the transfer of the acyl moiety to ghrelin was identified as ghrelin *O*-acyltransferase (GOAT). GOAT is a membrane-bound acyltransferase, specific for the acyl-modification of ghrelin. Interestingly, most ghrelin in the stomach is modified by *n*-octanoic acid; however, GOAT prefers to use *n*-hexanoic acid as the acyl donor rather than *n*-octanoic acid. The enzymes responsible for ghrelin processing, such as protease cleavage, acyl modification, and deacylation, have been identified and characterized in vivo and in vitro. The ghrelin-processing enzymes may be good targets for drug development to treat metabolic diseases and eating disorders.

Introduction

Ghrelin is an orexigenic peptide hormone that was originally isolated from rat stomach [1]. The name "ghrelin" is derived from "ghre," meaning "grow," to indicate the ability of this hormone to stimulate growth hormone (GH) release from the pituitary gland. Indeed, the two primary physiologic functions of ghrelin

M. Kojima, M.D., Ph.D. (✉)
Molecular Genetics, Institute of Life Science, Kurume University,
Hyakunenkohen 1-1, Kurume, Fukuoka 839-0864, Japan
e-mail: kojima_masayasu@kurume-u.ac.jp

K. Kangawa, Ph.D.
National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan

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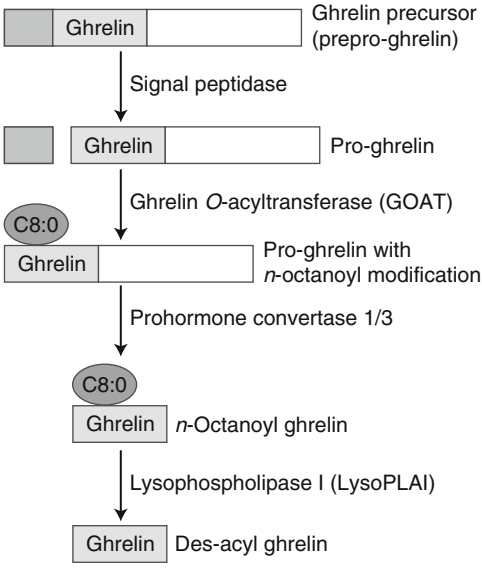

Fig. 2.1 Proposed processing steps for ghrelin. After the signal peptide is removed, the ghrelin precursor is modified by GOAT with acyl acid (primarily *n*-octanoic acid), and cleaved by the processing protease PC1/3 to produce active ghrelin. The acyl moiety is removed by lysophospholipase I

are to stimulate GH release and increase appetite [2]. Ghrelin also exhibits cardiovascular effects [3], induces gastric movement and gastric acid secretion [4], suppresses sympathetic nerve output [5, 6], and regulates glucose metabolism [7, 8].

Ghrelin generally contains an acyl modification at the third amino acid, a serine residue (threonine in the bullfrog and edible frog) [9]. In fact, all ghrelin peptides that have been isolated to date are modified by fatty acids. The serine (or threonine in frogs) at residue 3 is modified with a medium-chain fatty acid, typically *n*-octanoic acid. Importantly, this modification is essential for the biological activity of ghrelin. The enzyme that transfers the fatty acid to ghrelin was identified in 2008, elucidating much of the processing pathway for acyl-modified ghrelin [10, 11].

This review discusses the pathway by which the ghrelin precursor is converted to the mature ghrelin peptide and the enzymes that are involved in this process (Fig. 2.1).

Mammalian Ghrelins

Since ghrelin was first isolated from human and rat stomach, the peptide has been identified in many mammalian species [9]. Ghrelin amino-acid sequences have been reported for American bison (GenBank Accession number: AH013663), bos taurus indicus (DQ118140), cat (NM_001009853), cow (AB035702), dog

Fig. 2.2 Comparison of mammalian ghrelin sequences. Identical amino acids are *shaded*. The *asterisk* indicates the acyl-modified serine residue at the third amino-acid position

(AB060700), giant panda (EU375448), goat (AH013721), golden hamster (EU863658), hamadryas baboon (DQ987858), horse (XM_001491134), house shrew (AB364508), human, Mongolian gerbil (AF442491), moose (AH013724), mouse (AB035701), mule deer (AY455994S1), pig (AB035703), pronghorn (AY454075), pygmy sperm whale (AH013719), rabbit (EF566009), rat, reindeer (AH013722), rhesus monkeys (AY371699), sheep (AB060699), spinifex hopping mouse (FJ843089), tammar wallaby (EU677468), wapiti (AH013723), water buffalo (DQ118139), and white-tailed deer (AY455987) (Fig. 2.2). The amino-acid sequences of mammalian ghrelins are well conserved; the seven N-terminal amino acids in particular are identical. The third amino acid is a serine residue, which is modified by a fatty acid and is essential for ghrelin activity in all of these mammalian peptides. Conservation of the sequence and the required acyl modification at the third residue suggest that ghrelin undergoes a precise series of processing steps.

Processing Proteases for Ghrelin Precursor

The amino-acid sequences of not only ghrelin peptides but also their precursors are well conserved. Mammalian ghrelin precursors contain the 28- or 27-amino-acid ghrelin peptide sequence immediately following a signal peptide. The cleavage sites for the signal peptide are the same in all mammalian ghrelin peptides.

Most bioactive peptides are processed by specific proteases that produce active forms of the peptides [12]. The typical processing signals are basic amino-acid pairs—i.e., Arg-Arg or Lys-Arg [13]. In the case of ghrelin, however, the C-terminal processing signal is a Pro-Arg sequence. Interestingly, in fish ghrelin peptides, the amino-acid sequences at the C-terminal processing sites are Gly-Arg-Arg, a typical processing signal for C-terminal amide conversion [14]. Indeed, the C-terminal amino acids in fish ghrelins are amidated [15, 16]. Unlike most C-terminal amide moieties in peptides, however, the amide structure is not necessary for ghrelin activity.

In the ghrelin precursor, the signal peptide is first cleaved to generate proghrelin. Then, the C-terminal Pro-Arg site is cleaved to produce the ghrelin peptide. Zhu et al. identified the processing protease that cleaves proghrelin at the C-terminal Pro-Arg site to produce the 28-amino-acid peptide [17]. The authors created prohormone convertase (PC) knockout mice and examined ghrelin in the stomach. The PC knockout mice lacked PC1/3, PC2, or PC5/6A. The gastric peptide fraction obtained from PC knockout mice was subjected to western blotting. The molecular weights of ghrelin were approximately 11 kDa in PC1/3 knockout mice and approximately 3.4 kDa in PC2 and PC5/6A knockout mice. These results suggest that proghrelin was not processed in the stomachs of PC1/3 knockout mouse. Moreover, immunohistochemical staining indicated that PC1/3 and ghrelin colocalize in A-like/X cells in the stomach, which are now called ghrelin cells. Thus, PC1/3 appears to be responsible for processing proghrelin into the ghrelin peptide.

In the pancreas, ghrelin was shown to colocalize with glucagon in islet α cells and is expressed in ε cells, a newly identified pancreatic cell type [18–20]. On the other hand, ghrelin did not colocalize with insulin in β cells. PC1/3 is expressed mainly in β cells, where the enzyme participates in pro-insulin processing [21]. Some but not all ghrelin-positive cells, however, express PC1/3 or PC2. These results suggest that proghrelin is not cleaved exclusively by PC1/3 in pancreas, in which PC2 or other processing proteases may contribute to proghrelin processing.

Hypothalamic ghrelin reportedly resembles gastric ghrelin [22]. More studies are necessary to determine whether PC1/3 is the only protease that processes ghrelin in the hypothalamus or other processing proteases, such as furin, are involved. Intriguingly, Takahashi et al. reported that coexpression of ghrelin and furin in cultured cells resulted in cleavage of proghrelin into ghrelin [23].

Identification of Ghrelin O-Acyltransferase

Ghrelin is the first and, at the present time, only peptide hormone that is known to be modified by a fatty acid. In fact, acylation of ghrelin is essential for the activity of the peptide [24], making the enzyme that catalyzes this reaction critical for ghrelin's activities. In 2008, the enzyme that catalyzes the transfer of the acyl moiety to ghrelin was identified as GOAT, clarifying the pathway by which the ghrelin precursor is processed into active ghrelin [10, 11].

Ghrelin *O*-acyltransferase (GOAT) was discovered independently by two research groups: one at the University of Texas Southwestern Medical School and another at Eli Lilly & Co. Led by the Nobel Prize winners Brown and Goldstein, the team at University of Texas Southwestern Medical School searched a genome database and found several orphan acyltransferases that had not been characterized. The authors expressed both ghrelin and the putative enzymes in a cell culture system and assessed the production of acyl-modified ghrelin. These experiments determined that MBOAT4 (membrane-bound *O*-acyltransferase) was responsible for acylation of the ghrelin peptide [11].

The group from Eli Lilly & Co. also identified several candidate acyltransferases from a database search, resulting in isolation of GOAT, the same MBOAT4 enzyme described by the group from Texas [10]. Small interfering RNA (siRNA) specific for GOAT was used to show that the enzyme is necessary for acylation of ghrelin in a medullary carcinoma cell line (TT cells), which expresses GOAT endogenously. GOAT-specific siRNA inhibited the production of acyl-modified ghrelin in these cells. Moreover, MBOAT4 knockout mice failed to produce acyl-modified ghrelin.

Together, these results show that GOAT is the acyltransferase that catalyzes the addition of an acyl acid to the hydroxyl group of the third residue serine in ghrelin. GOAT, a membrane-bound enzyme with multiple transmembrane domains, is a member of an acyltransferase family that comprises at least 16 enzymes [25]. Among them, only GOAT has been shown to acylate ghrelin. Of note, GOAT enzymes have been identified in mammals, birds, and fish.

Distribution of GOAT

The tissue distribution of GOAT is similar to that of ghrelin: GOAT is predominantly found in gastrointestinal organs, in particular the stomach, the main expression site of ghrelin [10, 11]. GOAT has also been detected in testes, the pituitary gland, pancreas, and plasma [26, 27]. A UniGene search for GOAT (MBOAT4) identified mice EST clones encoding GOAT in brain, pancreas, pituitary, and spinal cord tissues; ghrelin is also expressed in pituitary and pancreas tissues. In addition, localization of GOAT in plasma suggests that ghrelin may be acylated as it circulates [27].

Characterization of GOAT

GOAT functions optimally at pH 7–8 [28, 29], whereas the activity of the processing protease PC1/3 peaks in an acidic environment (pH 5–6) [13]. Secretory granules transition from neutral to acidic during prohormone processing, resulting in a pH of 5–6. The data indicate that the acyl-acid moiety is added to proghrelin and not after the peptide is cleaved to the 28-amino-acid ghrelin peptide. Zhu et al. confirmed this model by using western blotting to show that proghrelin in the stomach is modified by *n*-octanoic acid [17].

Fe^{3+} and Cu^{2+} inhibit GOAT activity, which was completely blocked at Fe^{3+} concentrations greater than 5 mM and Cu^{2+} concentrations greater than 0.5 mM. EDTA and EGTA had no effect on GOAT activity, indicating that the enzyme does not absolutely require cations [28].

Peptide Substrates for GOAT

GOAT modifies the serine at the third amino-acid residue, whereas the other serine residues in human ghrelin are not affected. Replacing the serine residues at the second, sixth, and 18th amino-acid positions in ghrelin with alanine did not alter *n*-octanoic acid modification of the third residue. Changing the third residue to an alanine, however, prevented GOAT from modifying the peptide [29]. The third amino-acid residue of frog ghrelin is a threonine, which is also modified using *n*-octanoic acid [30]. When the target serine in rat ghrelin was changed to threonine, GOAT modified the threonine residue using *n*-octanoic acid [11].

Interestingly, when the first amino acid (glycine) or fourth amino acid (phenylalanine) was changed to an alanine, the target serine was not acylated [29]. No acylation effects were observed after replacing the serine, leucine, serine, or proline at amino-acid positions 2, 5, 6, and 7, respectively, with alanine. In addition, adding two amino acids (Ser-Ala) to the N terminus significantly suppressed acylation of the serine at position 3. Thus, the glycine, serine, and phenylalanine at positions 1, 3, and 4, respectively, are important for ghrelin acylation.

Other studies examined various synthetic ghrelin N-terminal peptide substrates to determine the peptide length required for acylation by GOAT [28]. The authors determined that, at minimum, the N-terminal four amino acids of ghrelin were sufficient, although shorter synthetic peptides led to reduced acylation efficiency.

Acyl Donors for Ghrelin

GOAT is able to use a broad range of acyl-acid substrates when modifying ghrelin: from acetic acid (C2:0) to tetradecanoic acid (C16:0) [10]. Most gastric ghrelin, however, is modified by *n*-octanoic acid, whereas the other acylated

forms of ghrelin exist only at low levels. In addition, CoA-conjugated acyl acids rather than the free forms are used to modify ghrelin [28].

Most ghrelin in the stomach is modified by *n*-octanoic acid, although ghrelin peptides modified by *n*-hexanoic acid and *n*-decanoic acid have been detected [31, 32]. An in vitro assay revealed that GOAT prefers to use *n*-hexanoic acid as the acyl donor rather than *n*-octanoic acid [28]. In our previous studies, we showed that *n*-hexanoic acid ingestion significantly increased gastric concentrations of *n*-hexanoyl ghrelin in mice [33], supporting the preference of GOAT for *n*-hexanoic acid as an acyl donor.

The preference of GOAT for *n*-hexanoyl raises the question of why ghrelin in stomach exists primarily as the *n*-octanoyl form. One possibility is that ingested food may contain higher concentrations of *n*-octanoic acid than *n*-hexanoic acid, resulting in a higher concentration of *n*-octanoic acid in the stomach. Thus, the concentration of acyl acid in the stomach may determine how ghrelin is acyl-modified. Analyses of various ghrelin species and acyl-acid concentrations in food and stomach will provide an answer for this question.

Effects of Ingested Fatty Acids on Ghrelin Acylation

Ingested fatty acids can be directly used for ghrelin modification, which was shown using relatively rare *n*-heptanoic acid. Ingestion of *n*-heptanoic acid, which has seven carbon atoms, resulted in *n*-heptanoyl ghrelin in stomach and plasma [33, 34]. This form of ghrelin, which is not produced naturally, was secreted and circulated in blood. Thus, GOAT can catalyze acylation of ghrelin with *n*-heptanoic acid.

Ingested fatty acids can be used to acylate ghrelin in humans as well. Ashitani et al. examined plasma ghrelin concentrations in patients with cachexia related to chronic pulmonary disease after ingestion of nutrients containing *n*-octanoic acid [35]. Two hours after ingestion of *n*-octanoic acid, plasma concentrations of *n*-octanoyl ghrelin increased and remained high until the next day. On the other hand, plasma concentrations of desacylated ghrelin, which is not acylated, were similar to those observed in control subjects.

Kirchner et al. created transgenic mice that express both ghrelin and GOAT in liver cells [34]. The authors found that plasma concentrations of desacyl and C2 (acetyl) ghrelin increased, whereas the concentration of *n*-octanoyl ghrelin did not change. The results may reflect the low level of *n*-octanoic acid in the liver. In fact, when these transgenic mice were fed food containing C8-MCT, the plasma concentration of *n*-octanoyl ghrelin significantly increased. After ingestion of C8-MCT, the transgenic mice became heavier than wild-type (WT) mice, and the percentage body fat was higher in the transgenic mice. Moreover, energy consumption was lower in the transgenic mice compared with WT mice during both the light and dark phases of the day. Thus, ingested medium-chain fatty acids are directly used for ghrelin acylation and hence are important regulators of ghrelin activity.

GOAT Inhibitors

Peptides that mimic the five N-terminal amino acids of ghrelin (GSSFL-NH_2, GSAFL-NH_2, and GSSFL-COOH) inhibited *n*-octanoyl modification of ghrelin [29]. A similar peptide with an *n*-octanoyl modification (GSS(C8:0)FL-NH_2) also inhibited GOAT. Moreover, a peptide in which the third amino acid was changed to (S)-2,3-diaminopropionic acid (Dap) before modification with *n*-octanoic acid as well as [Dap]octanoyl-ghrelin (1–5)-NH_2 potently inhibited GOAT activity.

Recently, Barnett et al. described another potent synthetic inhibitor for GOAT, GO-CoA-Tat [36]. GO-CoA-Tat is a bisubstrate inhibitor that consists of three components: an octanoylated peptide containing the 10 N-terminal amino acids of ghrelin, coenzyme A, and a Tat peptide, an 11-amino-acid oligomer from the HIV Tat peptide to enhance cell penetration. Intraperitoneal injection of GO-CoA-Tat inhibited the production of acylated ghrelin and suppressed body weight gain. GO-CoA-Tat improves glucose tolerance, because it increases insulin concentrations and decreases glucose levels. These results indicate that GOAT inhibitors may provide clinical approaches for treating diabetes and metabolic diseases.

Phenotypic Analyses of GOAT Knockout Mice

GOAT knockout mice suggested that GOAT contributes to GH release and restriction of calorie intake [34, 37]. Four days on a restricted calorie diet resulted in 60% decreases in the body weights, percentage body fat, and blood glucose levels in both WT and knockout mice [37]. After day 4, however, blood glucose concentrations in WT mice were stable at 5,876 mg/dl, whereas GOAT knockout mice showed additional decreases to 1,236 mg/dl on day 7. Under these conditions, WT mice showed normal activity levels, whereas GOAT knockout mice were lethargic and began to die. Moreover, the WT mice showed increased plasma GH concentrations, an effect that was not as pronounced in the GOAT knockout mice. Ghrelin or GH injections into the GOAT knockout mice restored the normal blood glucose concentration and rescued the mice from death. These results suggest that, in a state of severe calorie restriction, ghrelin increases GH concentrations and maintains the blood glucose concentration. It is not clear whether ghrelin or ghrelin receptor (GHS-R) knockout mice show the same phenotypes.

Molecular Forms of Acylated Ghrelin

As stated previously, most mammalian and nonmammalian ghrelin is modified with *n*-octanoic acid. Several minor forms of acylated ghrelin have been isolated from various species (Table 2.1).

Table 2.1 Minor forms of acylated ghrelin isolated from various species

Species	Fatty acids of ghrelin acyl-modification	Reference
Human	C8:0	Hosoda et al. [31]
	C10:0	
	C10:1	
Cat	C8:0	Ida et al. [32]
	C10:0	
	C10:1	
	C10:2	
	C13:0	
	C13:1	
Goat	C8:0	Ida et al. [38]
	C8:1	
	C9:0	
	C10:0	
Chicken	C8:0	Kaiya et al. [16]
	C10:0	
Rainbow trout	C8:0	Kaiya et al. [40]
	C8:1	
	C10:0	
	C10:1	
	C10:2	
Eel	C8:0	Kaiya et al. [15]
	C8:1	
	C10:0	
	C10:1	
Tilapia	C10:0	Kaiya et al. [41]
	C10:1	
Channel catfish	C10:0	Kaiya et al. [42]
	C10:1	
	C10:2	
Bullfrog	C8:0	Kaiya et al. [30]
	C10:0	
Red-eared slider turtle	C8:0	Kaiya et al. [42]
	C10:0	
	C10:1	

Human stomach contains at least three types of acylated ghrelin: octanoylated (C8:0), decanoylated (C10:0), and decenoylated (C10:1) [31]. In the cat stomach, at least seven forms of acylated ghrelin have been observed: C8:0, C8:1, C10:0, C10:1, C10:2, C13:0, and C13:1 [32]. The caprine stomach contains at least four types of acylated ghrelin: C8:0, C8:1, C9:0, and C10:0 [38]. Among birds, chicken ghrelin is acylated by either *n*-octanoic or *n*-decanoic acid [39].

Fish ghrelins are also modified by several acyl species: the serine residue at position 3 of rainbow trout and eel ghrelins can be modified by octanoic acid, decanoic acid, or unsaturated forms of those fatty acids [15, 40]. The serine residue

at position 3 in tilapia ghrelin has been shown to be modified by *n*-decanoic acid or decenoic acid [41]. Decanoyl modification, however, generates the major form of ghrelin in tilapia, whereas octanoylated ghrelin levels are very low. Ghrelin from channel catfish (*Ictalurus punctatus*) show third residue modifications with *n*-decanoic acid (C10:0) or different unsaturated fatty acids, such as C10:1 and C10:2 [42].

The third residue of bullfrog ghrelin is a threonine rather than the serine residue observed in mammalian ghrelins [30]. Serine and threonine both possess hydroxyl groups, and can be modified by fatty acids. The threonine at position 3 in bullfrog ghrelin is modified by either *n*-octanoic or *n*-decanoic acid. Ghrelin has also been purified from the stomach of the red-eared slider turtle (*Trachemys scripta elegans*) [43]. Trachemys ghrelin is a 25-amino-acid peptide that is acylated at the third residue (serine) by *n*-octanoic (C8:0), decanoic (C10:0), or unsaturated decanoic acid (C10:1).

Although there are multiple forms of acylated ghrelin in vertebrates, only one GOAT enzyme has been identified in each genome sequence. These results indicate that GOAT is able to modify ghrelin using several different medium-chain fatty acids. In fact, in vitro studies confirmed a broad substrate specificity for GOAT. Moreover, fatty acids used for ghrelin acylation may reflect species-specific diets.

O-Glycosylation of Ghrelin in the Red Stingray

A unique form of a ghrelin-like peptide was detected in the cartilaginous red stingray (*Dasyatis akajei*) [44]. The peptide is *O*-glycosylated with mucin-type glycan chains at the threonine at position 11 with or without a similar moiety on the serine residue at position 10. The serine residue at position 3 is also modified with *n*-octanoic acid. Removing the modified glycan structure attenuated the activity of this peptide.

Enzymes That Catalyze Ghrelin Deacylation

Deacylation is also important for regulating ghrelin activity. Several enzymes have been proposed as candidates for ghrelin deacylation, including paraoxonase [45], carboxylesterase [46], butyrylcholinesterase [46], and other esterases.

Recently, acyl protein thioesterase I/lysophospholipase I (LysoPLA I) was reported to catalyze ghrelin deacylation [47, 48]. Shanado et al. purified an enzyme from stomach homogenate that catalyzed ghrelin deacylation [47]. The enzyme was identified as LysoPLA I and recombinant LysoPLA I was shown to deacylate ghrelin potently. Satou et al. purified LysoPLA I as a ghrelin deacylation enzyme from fetal bovine serum and the culture medium of HepG2 cells [48]. LysoPLA I, however, also catalyzes hydrolation of 2-lysophosphatidylcholine to glycerophosphocholine and carboxylate, so it appears not to be specifically involved in ghrelin deacylation.

Interestingly, including long-chain fatty acyl CoA in the ghrelin acylation reaction stimulated the formation of acylated ghrelin [29]. This reaction was accelerated by the addition of myristoyl-CoA, palmitoyl-CoA, or oleoyl-CoA, but not by free myristate. Long-chain fatty acyl CoAs are thought to serve as a decoy substrate for ghrelin-deacylating enzymes, thereby increasing the concentration of acylated ghrelin.

Conclusion

The enzymes responsible for ghrelin processing, such as protease cleavage, acyl modification, and deacylation, have been identified and characterized in vivo and in vitro. Among these enzymes, GOAT catalyzes acylation of ghrelin. Other than GOAT, 15 enzymes that contain putative catalytic domains with acyl-transferase activity have been identified from a mouse genomic database [11, 25]. Among these enzymes, seven putative MBOATs—including splicing variants: MBOAT1-a/b, MBOAT2-a/b, MBOAT5, LRC4, and GUP1—are orphan acyltransferases and their substrates have yet to be identified. These results suggest that there may be other peptide hormones or signaling molecules with acyl modifications. Importantly, the ghrelin-processing enzymes may be good targets for drug development to treat metabolic diseases and eating disorders [36]. Further studies will likely provide novel therapeutic approaches based on ghrelin processing.

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Chapter 3
Ghrelin's Novel Signaling in Islet β-Cells to Inhibit Insulin Secretion and Its Blockade As a Promising Strategy to Treat Type 2 Diabetes

Katsuya Dezaki, Boldbaatar Damdindorj, Tomoyuki Kurashina, and Toshihiko Yada

Abstract Ghrelin, an acylated 28-amino acid peptide, was isolated from the stomach and circulating ghrelin is produced predominantly in the oxyntic mucosa of stomach. In addition to its unique role in regulating mealtime hunger and lipid metabolism, we here review the physiological role of ghrelin in the regulation of insulin release and glucose metabolism. Ghrelin is expressed in pancreatic islets and released into pancreatic microcirculation. Ghrelin inhibits insulin release in mice, rats, and humans. The signal transduction mechanisms of ghrelin in islet β-cells are very unique, being distinct from those utilized for growth hormone release. Pharmacological and genetic blockades of islet-derived ghrelin markedly augment glucose-induced insulin release in vitro. In high-fat diet-induced mildly obese mice, ghrelin-deficiency enhances insulin release and prevents impaired glucose tolerance. Thus, manipulation of insulinostatic function of ghrelin–growth hormone-secretagogue receptor system, particularly that in islets, could optimize the amount of insulin release to meet the systemic demand, providing a potential therapeutic application to prevent type 2 diabetes.

Introduction

Circulating ghrelin is produced predominantly in the stomach [1]. Ghrelin in a lower amount is also detected in the intestine, pancreas, kidney, immune system, placenta, testis, pituitary, lung, and hypothalamus [2–14]. Growth hormone (GH)-secretagogue receptor (GHS-R), which is recognized as the ghrelin receptor, is expressed in a

K. Dezaki, Ph.D. (✉) • B. Damdindorj, M.D., Ph.D. • T. Kurashina, M.D. • T. Yada, Ph.D.
Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
e-mail: dezaki@jichi.ac.jp; tyada@jichi.ac.jp

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variety of central and peripheral tissues including the hypothalamus and pituitary [9, 11, 15–22]. Ghrelin is a potent stimulator of GH release [2, 23–27]. Ghrelin also stimulates feeding; ghrelin injected either centrally [28–31] or peripherally [29, 32] potently stimulates food intake. In humans, ghrelin peaks before meals, suggesting its role as a hunger signal [33, 34]. Ghrelin also promotes adiposity [35]. Cardiovascular actions of ghrelin have been reported [36–42]. Given this wide spectrum of biological activities, the discovery of ghrelin opened many new perspectives within neuroendocrine, metabolic, and cardiovascular research, thus suggesting its possible clinical application [43].

Ghrelin and GHS-R are also located in pancreatic islets [8, 9, 13, 44–48]. Ghrelin *O*-acyltransferase (GOAT), which has been identified as the enzyme that promotes the acylation of the third serine residue of ghrelin, is highly expressed in the pancreatic islets and β-cell line [49–51]. Ghrelin inhibits insulin release in mice, rats, and humans [45, 52–54]. Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance in humans [55, 56]. In addition, the plasma ghrelin level correlates inversely with obesity [57–59]. These early studies suggested that ghrelin could be involved in the regulation of insulin release and action, glucose metabolism, and energy metabolism.

Here we review the physiological role of ghrelin in the regulation of insulin release and glucose metabolism, and present a potential therapeutic avenue to manipulate ghrelin signaling and thereby counteract the progression of type 2 diabetes.

Systemic Effects of Ghrelin on Insulin Release and Glucose Metabolism

Systemic action of exogenous ghrelin to elevate blood glucose levels has been well documented in humans and rodents [45, 52, 60–62]. In mice fasted overnight, intraperitoneal (i.p.) administration of ghrelin at concentrations of 1 and 10 nmol/kg significantly elevated blood glucose levels at 30 min after administration [45]. The hyperglycemic effect of ghrelin was completely blocked by simultaneous administration of GHS-R antagonist, [D-Lys^3]-GHRP-6. Desacyl-ghrelin failed to significantly alter blood glucose levels. These results indicate that ghrelin elevates blood glucose via specific interaction with GHS-R. The following observations support that the ghrelin-induced hyperglycemia is neither caused by GH, a hyperglycemic hormone, nor by insulin resistance; ghrelin increased blood glucose in GH-deficient *little* mice and control wild mice in a similar manner, and in insulin tolerance test (ITT) i.p. injection of insulin lowered blood glucose levels in the ghrelin-administered and control mice in a similar manner. By contrast, when ghrelin at 1 and 10 nmol/kg was simultaneously injected with glucose in glucose tolerance test (GTT), the insulin responses were markedly attenuated and the glucose responses were larger in comparison to the control without ghrelin. It has recently been shown that in healthy humans ghrelin suppresses insulin secretion and elevates

blood glucose in intravenous GTT [63]. Collectively, ghrelin elicits hyperglycemic effect in rodents and humans primarily by suppressing insulin release and independently of GH release and insulin resistance.

In fasted mice, i.p. administration of specific GHS-R antagonists, [D-Lys^3]-GHRP-6 and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P [64], significantly reduced fasting blood glucose concentrations by 10–30 mg/ml at 30 and 60 min in a dose-dependent manner [45], suggesting that endogenous ghrelin is involved in the regulation of fasting blood glucose. In ob/ob mice, a genetic model of obesity due to leptin-deficiency, chronic treatment with GHS-R antagonists reduced blood glucose and increased insulin levels [64]. In mice with GTT, insulin responses at 10 and 15 min were markedly enhanced and increases in plasma glucose at 30 and 60 min were markedly attenuated by simultaneous injection of [D-Lys^3]-GHRP-6 [45]. In ITT studies, i.p. injection of insulin lowered blood glucose levels similarly in the ghrelin receptor antagonist-injected and control mice [45]. Esler et al. [65] confirmed it by reporting that oral administration of a novel small molecule GHS-R antagonist improved glucose tolerance in rats by stimulating insulin secretion while eliciting no apparent effect on insulin sensitivity. Pretreatment of mice with GOAT inhibitor also showed increased insulin response and a reduced blood glucose in GTT [66].

In ghrelin-knockout (Ghr-KO) mice, plasma ghrelin levels were undetectable. When fed standard chow, no significant differences between male Ghr-KO and wild-type mice were observed at 8 weeks of age in body weights, total 24-h food intake, and blood glucose levels in fed states, confirming previous reports on Ghr-KO mice [67–70]. Behind these observations, a yet-unknown compensatory mechanism could have occurred in the knockout mice to maintain homeostatic regulation, since feeding, insulin release, and blood glucose levels are the life-saving functions. In GTT, Ghr-KO mice exhibited markedly enhanced insulin responses and attenuated glucose responses [71, 72]. The profiles of ITT exhibited little differences [71] or slight changes [72] between Ghr-KO and wild-type mice. Thus, the suppressed glycemic responses to GTT in Ghr-KO mice primarily result from enhanced insulin secretion, though possible additional effects of ghrelin on glucose production [73] or insulin sensitivity [72, 74] cannot be disregarded.

Ghrelin Is Expressed and Released in the Pancreatic Islets

Immunohistochemistry with antiserum against ghrelin demonstrated the immunoreactivity for ghrelin in a fraction of human and rat islet cells, which were observed mainly in the periphery of islets. In addition, GOAT is highly expressed in the pancreatic islets and INS-1 insulinoma cells [49–51]. Ghrelin-immunoreactive cells highly overlapped with glucagon-immunoreactive cells [8, 45, 75], while some of glucagon-immunoreactive cells were not immunoreactive to ghrelin. Immunoreactive ghrelin was also observed in mouse islets [76, 77]. Multiple experimental systems have shown ghrelin-immunoreactivity in α-cells [8, 45, 78,

79], β-cells [13, 79, 80], and novel islet cells [44, 47, 48] including those named ε-cells [46, 77]. It was also reported that ghrelin is expressed together with glucagon or pancreatic polypeptide in immature islet cells in rats [47]. Regarding receptors for ghrelin in islets, double immunohistochemistry revealed that GHS-R-like immunoreactivity mainly colocalized extensively with glucagon-immunoreactivity and partly with insulin-immunoreactivity in rat pancreatic islets [75], indicating expression of GHS-R in α- and β-cells. Messenger RNAs (mRNAs) encoding ghrelin and GHS-R are expressed in the pancreas of rats and humans [2, 8, 9, 13] as well as in β-cell lines [47, 80, 81]. The expression of pancreatic ghrelin changes dramatically during fetal development. Ghrelin mRNA and total ghrelin in the pancreas are markedly elevated selectively in the perinatal stages, at which their levels are 6–7 times greater than those in the fetal stomach [82]. It is suggested that ghrelin is expressed and located in different islet cell types depending upon the species, age, and conditions of animals/humans. Furthermore, release of ghrelin from pancreatic islets was assessed by comparing the ghrelin level in the pancreatic vein (splenic vein) with that in the pancreatic artery (celiac artery) in anesthetized rats (Fig. 3.1a). The concentrations of both acylated-ghrelin and desacyl-ghrelin in the pancreatic vein were significantly higher (about eight times and three times, respectively) than those in the pancreatic artery in rats (Fig. 3.1b), indicating that ghrelin is released from pancreas (Fig. 3.1c) [71]. Regulation of ghrelin release from the pancreas as compared to that from the stomach is an important issue that remains to be clarified.

Insulinostatic Function of Islet-Derived Ghrelin

In isolated rat islets, GHS-R antagonists ([D-Lys^3]-GHRP-6 and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P) markedly increased insulin release in the presence of glucose at 5.6 mM or higher and this response was abolished in the absence of external Ca^{2+} [45], indicative of Ca^{2+}-dependent insulin release by the receptor antagonists. Furthermore, antiserum against ghrelin, but not nonimmune serum, increased insulin release. These results suggest that endogenous ghrelin suppressed Ca^{2+}-mediated insulin release. Administration of exogenous ghrelin at relatively high concentration of 10 nM, but not 0.1 nM and 1 pM, attenuated 8.3 mM glucose-induced insulin release in islets, while it had no effect on basal insulin release at 2.8 mM glucose [45]. The similar concentration- and glucose-related inhibitory effects of exogenous ghrelin on insulin release in vitro are reported in the rat [81] and mouse islets [54]. This effective concentration of ghrelin around 10 nM is higher than the range of circulating ghrelin at 100 pM to 3 nM [59, 83]. However, it may be reasonable that the islet-derived ghrelin acts on islet β-cells at relatively higher concentrations, since the concentration at which a hormone works in a paracrine/autocrine manner is higher than that in an endocrine manner. On the other hand, 10 nM ghrelin failed to alter glucagon release from rat islets at both 2.8 and 8.3 mM glucose, and glucagon release at 5.6 mM glucose was not significantly

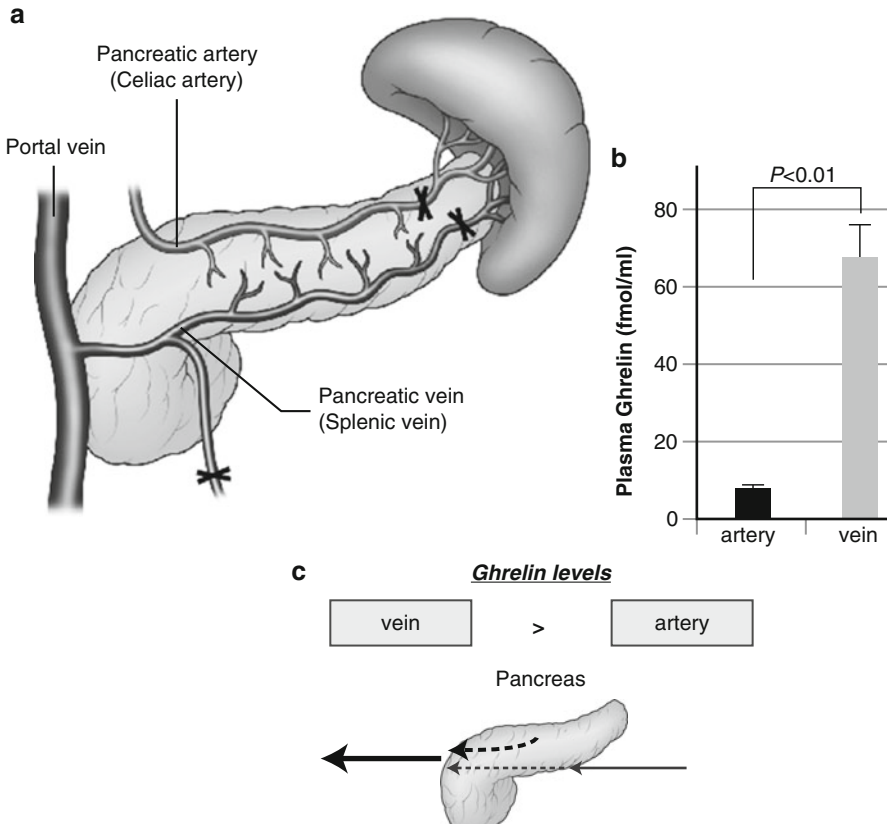

Fig. 3.1 Ghrelin release from pancreas. (**a**) To measure plasma ghrelin concentrations, blood samples were collected from the pancreatic arteries (celiac artery) and veins (splenic vein) of anesthetized rats. To avoid inflow of ghrelin from intestine and stomach to the splenic vein, the inferior mesenteric vein and spleen side of the splenic vein—including the short gastric and left gastroomental veins—were ligated (as denoted with ×). (**b**) The ghrelin level is higher in the pancreatic vein than in the artery, indicating that ghrelin is released from pancreas (**c**). "Copyright © 2008 Elsevier Inc. From Pharmacology & Therapeutics®, vol 118, 2008; 239–249. Reprinted with permission from Elsevier Inc. in slightly modified form"

altered by 1 μM [D-Lys^3]-GHRP-6 [45]. These results suggest that neither exogenous nor endogenous ghrelin regulates glucagon release.

To examine physiological roles of the pancreatic islet-produced ghrelin, we employed insulin release from the perfused rat pancreas, an in vitro system that retains the intact circulation in pancreatic islets while excluding the influence of other organs [84]. A rise in the perfusate glucose concentration from 2.8 to 8.3 mM evoked insulin release in a biphasic manner. Both the first and second phases of glucose-induced insulin release were significantly enhanced by blockade of GHS-R with a GHS-R antagonist [D-Lys^3]-GHRP-6, and by immunoneutralization of

endogenous ghrelin with anti-ghrelin antiserum [71]. Conversely, administration of exogenous ghrelin (10 nM) suppressed both phases of glucose-induced insulin release [53, 71]. None of these treatments affected basal insulin release at 2.8 mM glucose. These findings indicate that the endogenous ghrelin suppresses glucose-induced insulin secretion within islets [85, 86]. It should be noted that the effect of GHS-R antagonist could be partly due to blockade of constitutive activity of GHS-R [87, 88].

The role of islet-derived ghrelin in insulin release has also been demonstrated by the analysis in ghrelin-deficient mice. Morphological analysis of pancreatic sections showed that the density and average size of islets were not altered in wild-type mice as compared to Ghr-KO mice [71]. The number and size of isolated islets obtained by collagenase digestion were not altered in Ghr-KO mice. Glucose (8.3 and 16.7 mM)-induced insulin release from isolated islets of Ghr-KO mice was significantly greater than that of wild-type mice, while basal levels of insulin release at 2.8 mM glucose were not altered. No difference was observed between Ghr-KO and wild-type mice in insulin content per islet and mRNA expressions of insulin 1 and insulin 2 [71]. Collectively, the larger amount of insulin release in islets of Ghr-KO mice results from greater insulin secretory response to glucose, while insulin production is unaltered. Barnett et al. [66] reported that pretreatment of human islet cells with a peptide-based GOAT inhibitor promoted significant increase in insulin response to a glucose challenge. These results indicate that GOAT may catalyze acylation of ghrelin in islets and confirm that the insulinostatic function of islet-derived acyl-ghrelin suppresses insulin response in human.

Ghrelin Signaling Mechanisms in Islet β-Cells

Pertussis Toxin-Sensitive GTP-Binding Protein

Insulinostatic effects of endogenous and exogenous ghrelin were blunted in islets isolated from rats pretreated with pertussis toxin (PTX), a specific inhibitor of Gi and Go subtypes of trimeric GTP-binding proteins [89] that function in islet β-cells [90], while addition of 25 mM KCl enhanced insulin release from these islets [91].

The enhancement of glucose-induced insulin release from islets of Ghr-KO mice was undetectable after treatment with PTX [91], indicating that endogenous ghrelin attenuates insulin release via PTX-sensitive G proteins. PTX, an inhibitor of $G_{i/o}$ subtypes of trimeric G-proteins, was formally recognized as the islet-activating protein (IAP), because this agent increased insulin release from isolated islets [89]. The effect of IAP has suggested the presence of intra-islet substance that activates $G_{i/o}$-protein-coupled receptors to decrease insulin release. However, the endogenous islet substance whose insulinostatic action is antagonized by PTX/IAP is as yet unidentified. The insulinostatic effect of endogenous ghrelin in islets, as evidenced by enhanced insulin release due to ghrelin gene-knockout and

GHS-R antagonist, was blunted by PTX treatment. The enhancement of insulin release by ghrelin gene-knockout or GHS-R antagonist was as large as 70–80% of the enhancement by PTX/IAP treatment [91]. These findings indicate that a large part, but not all, of the PTX/IAP effect is exerted by blocking the action of endogenous ghrelin in islets. The apparently ghrelin-independent portion of the PTX/IAP effect could be due to inhibition of the signaling of somatostatin, an islet hormone that is released from δ-cells and inhibits insulin release via PTX-sensitive G-proteins [90], although a paracrine role for somatostatin in islets has long been controversial [92].

Voltage-Dependent Kv Channel

At substimulatory glucose concentrations, β-cells maintain the resting membrane potential at a hyperpolarized level of around −70 mV. Elevation of the blood glucose concentration increases glucose uptake and metabolism by β-cells, resulting in closure of the ATP-sensitive K^+ (K_{ATP}) channels. When K^+ efflux is reduced, inward currents more effectively contribute to the membrane potential and depolarize the membrane, inducing bursting spikelike short action potentials at membrane potentials positive from −50 to −40 mV. These action potentials are produced by orchestrated openings of voltage-dependent Ca^{2+} channels and voltage-gated K^+ channels. Under the condition of nystatin-perforated whole-cell current-clamp mode, glucose (8.3 mM) elicited firings of action potentials in rat β-cells. The firings were characterized by spikelike and repetitively occurring action potentials on top of the plateau phase of slow waves [91]. These electrical firings were attenuated by ghrelin (10 nM) in a reversible manner. Ghrelin decreased both the frequency and amplitude of the firings, while mean membrane potentials measured at most repolarized levels between slow-wave potentials at 8.3 mM glucose were not significantly altered. These findings suggest that the ghrelin does not hyperpolarize the membrane potential but decreases the activity of action potentials in β-cells.

Voltage-dependent potassium channels are involved in repolarization of excitable cells. In pancreatic β-cells, activation of delayed rectifier K^+ (Kv) channels repolarizes cells and attenuates glucose-stimulated action potentials, thereby limiting Ca^{2+} entry through voltage-dependent Ca^{2+} channels to suppress insulin secretion [93]. It has recently been reported that the Kv channels in pancreatic β-cells are regulated by glucose metabolism and that the metabolic regulation of the Kv channel current is voltage dependent [94, 95]. Blockade of Kv channels can promote glucose-dependent insulin secretion [96–98]. In a study using perforated whole-cell clamp, ghrelin increased the amplitudes of K^+ currents in rat single β-cells in a reversible manner [45, 91]. This response occurred in the presence of tolbutamide, a blocker of K_{ATP} channel. Current–voltage relations of this current depicted that ghrelin activated the outward Kv currents at the potentials positive to −30 mV. The ghrelin enhancement of Kv currents in the entire range of potentials was blunted in β-cells treated with PTX [91]. These results indicate that

PTX-sensitive G-proteins are required for ghrelin activation of Kv currents. This enhancing effect was blunted when 10 mM tetraethylammonium (TEA), a nonselective blocker of delayed-rectifier K^+ channels, was administered during exposure to ghrelin. Furthermore, in the presence of TEA, the ability of ghrelin to inhibit glucose-induced $[Ca^{2+}]_i$ increase [45] and insulin release [91] was partially but significantly diminished, suggesting that the enhancement of Kv channel conductance is linked to insulinostatic action of ghrelin. Moreover, stromatoxin, a specific blocker of the 2.1 subtype of Kv channels [99, 100], potentiated glucose-induced insulin release, and in the presence of stromatoxin ghrelin failed to attenuate glucose-induced insulin release [91]. These results suggest that ghrelin inhibits $[Ca^{2+}]_i$ increases and insulin release partly via the enhancement of the TEA-sensitive Kv current passing through the stromatoxin-sensitive Kv2.1 channels. Kv2.1 is expressed at high levels in islets of various species [96, 97], and immunohistochemical analysis shows expression of Kv2.1 specifically in β-cells of islets [101]. Taken together, an increase in Kv2.1 channel conductance mediates ghrelin-induced inhibition of insulin release as well as Ca^{2+} signaling.

Ghrelin did not potentiate the Kv currents in the presence of membrane-permeable cyclic AMP analogue dibutylyl-cyclic AMP, suggesting that ghrelin activation of Kv channels is mediated by cyclic AMP signaling pathway. It is known that members of the $G\alpha_i$-family are involved in linking inhibitory hormones and neurotransmitters to cyclic AMP production [102–104], voltage-dependent Ca^{2+} channels [105, 106], and insulin exocytosis [90]. Whether the ghrelin action also involves one or all of these processes is unknown and requires further studies. Possible differences in the coupling of G proteins to signaling pathways between ghrelin and other inhibitory hormones, norepinephrine and somatostatin, also remain to be clarified.

K_{ATP} channel is the key molecule that determines resting membrane potentials and converts the glucose metabolism to the membrane excitation in β-cells [107, 108]. This channel, however, may not be the target for ghrelin signaling for the following reasons: membrane potentials which are mainly controlled by K_{ATP} channels were not significantly changed by ghrelin, and K_{ATP} channel currents at 8.3 mM glucose conditions were not altered by ghrelin application [91]. It appears that the ghrelin-induced decrease in the action potential firing is caused largely, if not completely, by activation of Kv channels. Thus the current consensus is that ghrelin activates voltage-dependent Kv channels via PTX-sensitive mechanisms to rapidly repolarize the membrane and shorten bursting action potentials, leading to attenuation of glucose-induced increase of and oscillations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in β-cells and thereby insulin secretion (Fig. 3.2) [85].

Calcium Signaling

In islet β-cells, $[Ca^{2+}]_i$ is considered the major regulator of insulin secretion [109, 110]. The role of the endogenous ghrelin in regulation of $[Ca^{2+}]_i$ in islet β-cells was explored by measuring $[Ca^{2+}]_i$ in whole islets by fura-2 fluorescence imaging. $[Ca^{2+}]_i$

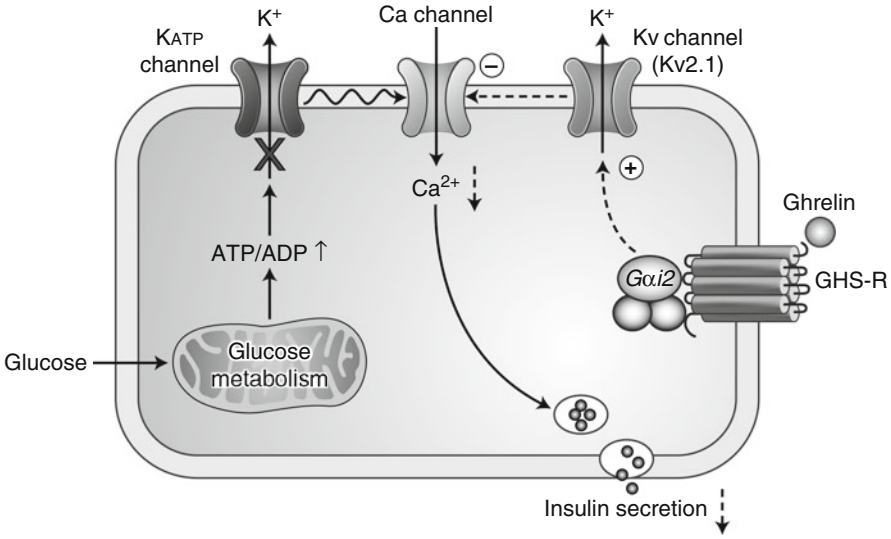

Fig. 3.2 Ghrelin signaling in islet β-cells. Closure of ATP-sensitive K^+ (K_{ATP}) channels by increases in ATP/ADP ratio following glucose metabolism induces membrane depolarization and increase in cytosolic Ca^{2+} concentrations via voltage-dependent Ca^{2+} channels, leading to insulin secretion in β-cells. Ghrelin activates β-cell GHS-R that is coupled with PTX-sensitive heterotrimeric G-protein $G\alpha_{i2}$, attenuates membrane excitability via activation of voltage-dependent K^+ channels (Kv2.1 subtype), and consequently suppresses Ca^{2+} influx and insulin release. "Copyright © 2008 Elsevier Inc. From Pharmacology & Therapeutics®, vol 118, 2008; 239–249. Reprinted with permission from Elsevier Inc. in slightly modified form"

in a whole islet was elevated mildly by increasing glucose concentration from 2.8 to 5.6 mM. In the presence of [D-Lys^3]-GHRP-6 that antagonizes the effect of endogenous ghrelin, the peak of the first-phase $[Ca^{2+}]_i$ response was enhanced and, in some islets, oscillations of $[Ca^{2+}]_i$ were induced [45]. The peaks and integrated $[Ca^{2+}]_i$ increases of the first-phase $[Ca^{2+}]_i$ responses to 5.6, 8.3, 16.7, and 22.4 mM glucose were all increased by the antagonist. Similarly, antiserum against ghrelin enhanced the $[Ca^{2+}]_i$ response to 8.3 mM glucose [45]. These results indicate that the endogenous ghrelin in islets restricts glucose-induced $[Ca^{2+}]_i$ increase in β-cells and thereby insulin secretion, presumably via a paracrine and/or autocrine route.

In rat single β-cells, ghrelin at a relatively high concentration of 10 nM, but not 0.1 nM, markedly suppressed the peak of the first-phase $[Ca^{2+}]_i$ responses to 8.3 mM glucose and this effect was blocked by GHS-R antagonist [45, 91]. Ghrelin preincubated with antiserum against ghrelin failed to affect $[Ca^{2+}]_i$ responses, confirming that the antiserum employed in our study neutralizes the activity of ghrelin. Ghrelin at 10 nM also attenuated $[Ca^{2+}]_i$ oscillations during the second-phase responses to 8.3 mM glucose in oscillating β-cells. The attenuation of $[Ca^{2+}]_i$ oscillations by ghrelin was abolished in the presence of GHS-R antagonist [45]. The effects of ghrelin on the first-phase and second-phase $[Ca^{2+}]_i$ responses were

abolished in β-cells following exposure to PTX. In contrast, 10 nM ghrelin had no significant effect on the $[Ca^{2+}]_i$ responses to 300 μM tolbutamide and 10 μM acetylcholine (ACh) at 2.8 mM glucose, indicating a selective action of ghrelin toward glucose [45].

Expression study revealed that GHS-R is coupled to G_{11}-phospholipase C (PLC) signaling, leading to production of IP_3 and Ca^{2+} release from IP_3-sensitive stores [15]. Synthetic GHSs and ghrelin evoke GH release via activation of PLC-mediated $[Ca^{2+}]_i$ increases in pituitary cells [111–115]. However, ghrelin attenuation of insulin release appears not to be mediated by PLC signaling pathways, since PLC activation operated by muscarinic ACh receptor for parasympathetic nerve input [116, 117] and GPR40 receptors specific for free fatty acids [118, 119] potentiates, but not attenuates, glucose-induced increases in $[Ca^{2+}]_i$ and insulin release in β-cells [117, 120, 121]. Thus, ghrelin may elicit different signaling pathways in a tissue-specific manner, which may underlie the opposing effects of ghrelin on GH release and insulin release. Heterotrimeric G proteins composed of α-, β-, and γ-subunits function as signal transducers that link the membrane receptor activation to intracellular effectors. PTX specifically ADP-ribosylates the α-subunit of G_i family of G-proteins ($G\alpha_i$) and thereby abolishes its linkage to G-protein-coupled receptors. The effects of ghrelin on insulin release, Kv channels, and $[Ca^{2+}]_i$ were all blocked by PTX, suggesting that certain subtypes of PTX-sensitive G-proteins are crucial in the ghrelin signal transduction in β-cells. Treatment of primary cultured rat β-cells with antisense oligonucleotide (AS) specific for the $G\alpha_{i2}$ subunit of G-proteins markedly decreased $G\alpha_{i2}$ subunit expression and knocked down the ability of ghrelin to attenuate $[Ca^{2+}]_i$ responses to glucose [91], whereas ASs specific for $G\alpha_{i1}$ and $G\alpha_{i3}$ had no effect on the inhibitory effect of ghrelin on $[Ca^{2+}]_i$ responses. Furthermore, in the β-cells treated with AS for $G\alpha_{i2}$, ghrelin failed to suppress glucose (8.3 mM)-induced insulin release [91]. These results indicate that $G\alpha_{i2}$-mediated signaling is crucial for the action of ghrelin to suppress glucose-induced $[Ca^{2+}]_i$ increase and insulin release and suggest that the ghrelin-induced attenuation of $[Ca^{2+}]_i$ increase is linked to that of insulin release. The finding that the G-protein $G\alpha_{i2}$ subtype mediates the ghrelin action is surprising in the light of the current concept that GHS-R signaling is mediated primarily by the G_{11} subtype [15]. However, our result is consistent with the reports that $G\alpha_{i2}$ is expressed in β-cells [90, 122] while $G\alpha_{11}$ is expressed primarily in non-β-cells in islets [123–125].

Ghrelin as Potential Therapeutic Target for Type 2 Diabetes

The enhanced insulin and suppressed glycemic responses to GTT in Ghr-KO mice could be beneficial under conditions of increased demand for insulin. When wild-type and Ghr-KO mice were fed high-fat diet (HFD) for 4 weeks, both mouse lines developed moderate increases in body weight to a similar extent [71]. In an apparent controversy, it was reported that another line of Ghr-KO mice were protected from a rapid weight gain during postweaning exposure to HFD for 3 weeks, which was

associated with decreased adiposity, increased energy expenditure, and increased locomotor activity as compared to wild-type mice [69]. HFD treatment significantly increased blood glucose levels in wild-type mice but not in Ghr-KO mice [71]. HFD treatment increased plasma insulin levels in wild-type mice, and this increment was much greater in Ghr-KO. Thus, Ghr-KO mice displayed a phenotype of enhanced insulin release and nearly normal glycemia under HFD conditions. This phenotype was even more prominent in GTT as follows. In wild-type mice, increases in blood glucose levels were exaggerated in HFD group compared to control diet group, exhibiting HFD-induced glucose intolerance. Although insulin response to GTT also tended to be enhanced in HFD group, the change was not statistically significant [71]. In Ghr-KO mice, in contrast, increases in blood glucose levels in HFD group were not significantly different from those of control diet group, and insulin response was markedly enhanced in HFD group (Fig. 3.3) [71]. Thus, ghrelin-deficiency promoted insulin release and prevented glucose intolerance in an HFD-induced obese model. Sun et al. [72] have reported that ablation of ghrelin in leptin-deficient ob/ob mice augmented insulin release and thereby markedly reduced hyperglycemia. Thus, the ghrelin blockade counteracts the obesity-associated glucose intolerance in both the life style-related and genetic obese models. As the underlying mechanism, we propose that lack of ghrelin and its insulinostatic activity increases the maximal capacity of glucose-induced insulin release and enables islets to secrete more insulin to meet an increased demand associated with obesity, thereby achieving normoglycemia (Fig. 3.4) [85, 86].

Doi et al. [126] have identified IA-2β as a ghrelin-induced gene by PCR-select subtraction method. IA-2β, also called phogrin, IAR, PTP-NP, or ICAAR, is an integral membrane glycoprotein that localizes to secretory granules in neuroendocrine tissues, which is also recognized as a β-cell autoantigen for type 1 diabetes [127–131]. IA-2β is a member of the receptor-type protein tyrosine phosphatase family. However, its phosphatase activity is inactive because of mutations at critical sites in the protein tyrosine phosphatase core domain, and site-directed mutagenesis can restore enzyme activity [132, 133]. It was shown that inhibition of IA-2β expression by the RNA interference technique ameliorated ghrelin's inhibitory effects on glucose-stimulated insulin secretion in MIN6 insulinoma cells [126]. Another interesting finding is that in ob/ob mice, ablation of ghrelin reduces the expression of uncoupling protein 2 (UCP2) [72]. In contrast, treatment of MIN6 cells with ghrelin for 1 h elevates expressions of UCP2 mRNA [134]. UCPs function to decrease metabolic efficiency by dissociating substrate oxidation in the mitochondrion from ATP synthesis. This is thought to be accomplished by promoting net translocation of protons from the intermembrane space, across the inner mitochondrial membrane to the matrix, thereby dissipating the potential energy available for conversion of ADP to ATP despite continued oxidation of fuels. This uncoupling effect of UCP2 then negatively regulates glucose-induced insulin release in β-cells [135–139]. UCP2 expression is also decreased by inhibition of posttranslational esterification of ghrelin. Islets isolated from mice treated with a GOAT inhibitor 24 h prior to isolation showed a 20-fold reduction in UCP2 mRNA levels as compared to islets isolated from control mice but no change in mRNA levels of insulin, ghrelin, and GHS-R

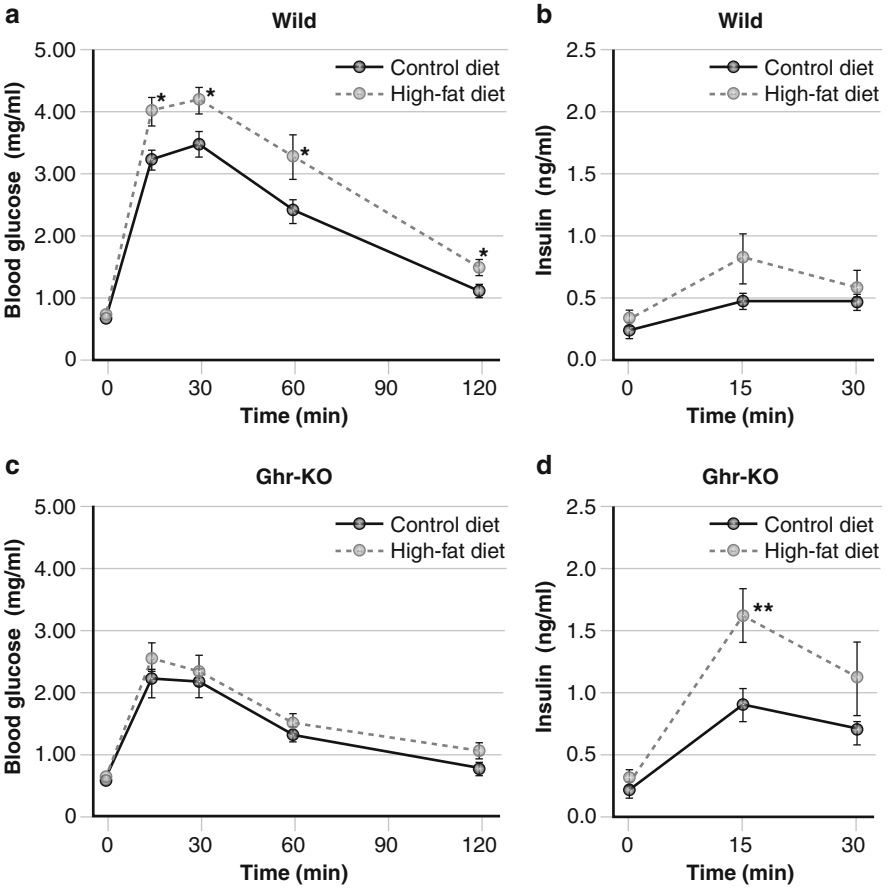

Fig. 3.3 High-fat diet-induced glucose intolerance is prevented in ghrelin-deficient mice. The mice were fed HFD or control diet from 8 to 12 weeks old. In wild-type mice, HFD group exhibited glucose intolerance (**a**) and slight enhancement of insulin release (**b**) during GTT (glucose 2 g/kg i.p.). In Ghr-KO mice, glycemic responses to GTT in HFD group were not different from those of control diet group (**c**), and insulin response at 15 min was markedly enhanced in HFD group (**d**). $^{*}P<0.05$; $^{**}P<0.01$ vs. control diet mice. "Copyright © 2006 American Diabetes Association From Diabetes®, vol 55, 2006; 3,486–3,493. Reprinted with permission from The American Diabetes Association"

[66]. Therefore, it is likely that ghrelin inhibits insulin release via two modes of action in β-cells: it acutely activates Kv channels and suppresses Ca^{2+} signaling, while chronically it may also upregulate UCP2 and IA-2β. In ob/ob mice, the increment of insulin release due to ghrelin-KO is remarkably large [72], which could be due to that UCP2 is upregulated in ob/ob mice β-cells [138] and therefore the magnitude of its inhibition by ghrelin-KO is greater. In fact, upregulation of UCP2 in the hypothalamic feeding center of ob/ob mice has recently been documented [140]. Moreover, the enhanced ghrelin action in ob/ob mice, assessed by the effect of

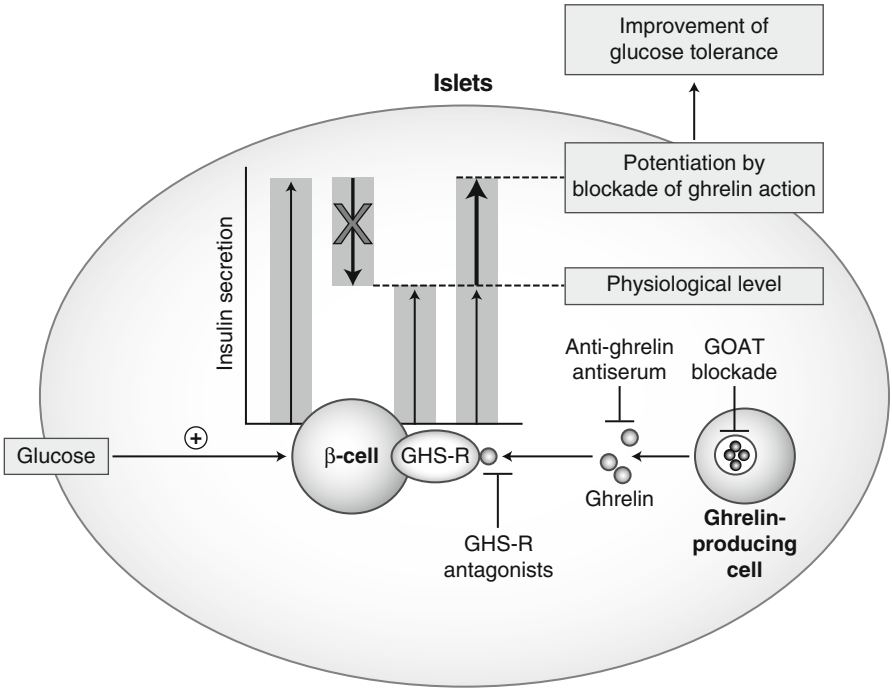

Fig. 3.4 Insulinostatic function of endogenous ghrelin in islets as therapeutic target for type 2 diabetes. The ghrelin of islet origin interacts with β-cell GHS-R in a paracrine/autocrine manner and attenuates glucose-induced insulin release, determining the physiological level of insulin release. Under conditions in which the systemic demand for insulin exceeds the physiological range, such as early stages of diet-induced obesity and/or insulin resistance, blockade of ghrelin function by GHS-R antagonists and anti-ghrelin antiserum can promote insulin secretion and improve glucose intolerance, providing a potential therapeutic avenue to counteract the progression of type 2 diabetes. "Copyright © 2008 Bentham Science Publishers Ltd. From Current Diabetes Reviews®, vol 4, 2008; 18–23 and © 2008 Elsevier Inc. From Pharmacology & Therapeutics®, vol 118, 2008; 239–249. Reprinted with permission from Bentham Science Publishers and Elsevier Inc. in slightly modified form"

ghrelin-deficiency, could be due to the lack of leptin, since leptin and ghrelin are considered mutual antagonists. To support it, plasma levels of ghrelin and leptin are inversely correlated [141]. Moreover, leptin counteracts the effects of ghrelin in several systems including the regulation of feeding and the neuropeptide Y neuron activity in the hypothalamic arcuate nucleus [142, 143]. It is worth noting that ghrelin, contrary to leptin, has several actions that could promote metabolic syndrome; it inhibits insulin release and elevates glycemia, stimulates feeding, and increases adiposity [35]. Therefore, suppression of these ghrelin actions could potentially counteract diabetes, hyperphagia, and obesity simultaneously, thereby acting as antimetabolic syndrome. However, it should be kept in mind that ghrelin also stimulates GH release [2], a factor that decreases the fat and increases the muscle mass.

The interplay between ghrelin and adipocytokines, such as leptin and adiponectin, and the impact of suppression of ghrelin–GHS-R system in the regulation of metabolism remain to be further elucidated.

Conclusion

Ghrelin, including that produced by pancreatic islets, plays a pivotal role in the regulation of insulin release and blood glucose in rodents and humans. Pharmacological, immunological, and genetic blockade of ghrelin expression or ghrelin action in pancreatic islets all markedly enhanced glucose-induced insulin release. These findings support the hypothesis that ghrelin is produced and released from pancreatic islet cells and act on islet β-cells via autocrine and/or paracrine manner, thereby suppressing glucose-induced insulin release. This function of ghrelin in regulating glucose metabolism, together with inducing GH release and feeding, suggests that ghrelin underlies the integrative regulation of energy homeostasis. When the systemic demand for insulin exceeds the physiological range, such as early stages of diet-induced obesity and/or insulin resistance, antagonism of ghrelin function can promote insulin secretion and prevent glucose intolerance, providing a potential therapeutic avenue to counteract the progression of type 2 diabetes [85, 86]. As predicted, oral administration of a novel GHS-R antagonist improved glucose tolerance in GTT in HFD rats and lean rats [65]. Since a single oral administration of the antagonist in fasted animals was sufficient to improve glucose tolerance, GHS-R antagonists appear to have a direct impact on glucose homeostasis independent of the potential additional benefits that may arise from chronic dosing [65].

It is of particular importance to clarify how ghrelin and its receptor in islets are regulated under physiological and pathological conditions, including fast/fed, lean/obese, and normoglycemic/diabetic states. Precise and relative roles of islet-derived vs. stomach-derived ghrelin in multiple steps of glucose metabolism remain to be further clarified. Ghrelin uniquely employs $G\alpha_{i2}$ and Kv channels, and further elucidation of the ghrelin signaling in islet β-cells could provide novel mechanisms for regulation of insulin secretion. Of particular interest is the effect of chronic augmentation and suppression of the ghrelin-ghrelin receptor system on the islet β-cell function, glucose and energy metabolism, which definitely requires further studies.

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Chapter 4
Gastric Ghrelin in the Regulation of Appetite and Metabolism

Luisa María Seoane, Ana Belen Crujeiras, Omar Al-Massadi, and Felipe F. Casanueva

Abstract Since its isolation 11 years ago, several groups have focused on elucidating the physiological actions of ghrelin. After its first action was described, namely, the regulation of GH secretion, ghrelin has been found to play additional roles in the regulation of energy balance. In addition to the initial measures of circulating ghrelin levels, the possibility of studying direct secretion of ghrelin from the stomach eliminating interferences with other organs uncovered an autonomous gastric mechanism regulating its own ghrelin secretion in order to adapt the organism to the different energetic requirement in each stage of life. This mechanism is sensitive to age-related changes, different hormonal factors, variations in nutritional status, and composition of the diet. Thus, ghrelin secreted from the stomach can contribute to the maintenance of energy homeostasis at different levels: at central level in order to regulate food intake, on adipose tissue, liver and muscle in order to affect adiposity and glucose metabolism, and finally it can act in a paracrine fashion in the stomach to interact with other gastrointestinal hormones regulating gastric emptying, motility, and finally energy balance.

L.M. Seoane, Ph.D.
Complejo Hospitalario Universitario de Santiago de Compostela/SERGAS, Santiago de Compostela, Spain

A.B. Crujeiras, M.D.
Department of Medicine, Complejo Hospitalario Universitario de Santiago (CHUS), Santiago de Compostela University, Santiago de Compostela, Spain

O. Al-Massadi, M.D.
Department of Medicine, Instituto Salud Carlos III, Complejo Hospitalario Universitario de Santiago (CHUS), CIBER de Fisiopatologia Obesidad y Nutricion (CB06/03), Santiago de Compostela University, Santiago de Compostela, Spain

F.F. Casanueva, M.D., Ph.D. (✉)
Department of Endocrinology and Nutrition, Universitario de Santiago de Compostela, Choupana sn, Santiago de Compostela 15780, Spain
e-mail: endocrine@usc.es

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Introduction

Ghrelin was isolated in 1999 from the stomach as an endogenous ligand for the growth hormone secretagogue (GHS) orphan receptor, opening up a new field in hormone research [1]. It was a typical example of reverse pharmacology [2]: first, an artificial GH releaser was developed, after which the growth hormone secretagogue receptor (GHS-R) was cloned, and finally, the endogenous ligand for this receptor (ghrelin) was isolated from the rat stomach [1].

Both rat and human ghrelin precursors for ghrelin are composed of 117 amino acids where the 28-amino acid active ghrelin sequence is immediately followed by the signal peptide [1]. In the rat stomach, two isoforms of mRNA encoding pro-ghrelin are produced from the gene by alternative splicing. One of them encodes the ghrelin precursor and the other encodes a precursor for des-Gln14-ghrelin. The enzyme responsible for pro-ghrelin processing in the stomach is the prohormone convertase 1/3 (PC1/3), which is responsible for the production of active ghrelin [3].

A total of 30% of circulating ghrelin are acetylated by *n*-octanoic acid at the serine^3 (Ser^3) residue; this is essential for its binding to the receptor GHS-R 1a. In addition, minor molecular forms of human ghrelin exist, including different types of acylation at Ser^3. Despite the extensive research carried out since the isolation of ghrelin 10 years ago, it was only in 2008 when the acyltransferase that catalyzes ghrelin octanoylation was identified as ghrelin O-acyltransferase (GOAT) [4]. This enzyme is expressed in the stomach and is colocalized with ghrelin in oxyntic mucosa [4]. At the gastric level, the primary action of GOAT is the acylation of ghrelin to produce the active form, which is able to bind GHS-R 1a to induce food intake, adiposity, and body weight gain. Indeed, GOAT knockout mice do not have the octanoylated ghrelin form and show increased levels of des-acyl ghrelin compared to wild-type mice. It has been found that GOAT is regulated by nutritional status [5], and the levels of the enzyme are highly influenced by the nature of the lipids in the diet [6].

Circulating ghrelin levels decrease by 65% after gastrectomy in humans [7] and by 80% in rodents [8], suggesting that the main source of ghrelin is the stomach. Ghrelin cells have been described to exist in a minor concentration along the entire gastrointestinal tract. The two primary types of cells producing ghrelin are the open-type cells of the stomach, which are regulated by luminal information, and the closed-type cells of the gut, which are regulated by hormonal and neural stimulation [9].

Although the stomach is the major focus of ghrelin production in the organism, lower expression has also been detected in other tissues such as the hypothalamus, testis, pituitary, ovary, heart, and placenta [10–15]. Additionally, using real-time PCR, ghrelin expression has been found to occur in the adrenal gland, adipocytes, gall bladder, skeletal muscle, myocardium, skin, spleen, liver, and prostate [16] although its biological significance is unclear.

Gastric Regulation of Ghrelin Secretion and GOAT mRNA Levels

The majority of published studies concerning the regulation of ghrelin secretion have been focused on plasma circulating ghrelin and ghrelin mRNA levels in the stomach. It has been assumed that serum plasma ghrelin is a direct reflection of ghrelin secretion by the stomach; however, this affirmation had not been proven. In 2007, we published a new organ culture model that enables the evaluation of direct ghrelin secretion from the stomach, avoiding the influence of other secretory tissues and problems of clearance. By using this model, the regulation of gastric ghrelin under different factors involved in the maintenance of energy balance was clarified [17].

Nutritional Stimuli Regulate Gastric Ghrelin Production

Ghrelin is thought to be a gastrointestinal hormone regulated by chronic or acute nutritional status changes in animals and humans and itself can regulate the nutritional behavior. Under conditions of food restriction, an elevation in plasma ghrelin levels is observed, while low levels of this peptide are found after feeding, thus suggesting that it may play a physiological role in hunger and meal initiation [18, 19], although this hypothesis was recently challenged [6]. Using the organ culture model [17], it was found that the regulation of food intake by plasma ghrelin starts at the gastric tissue, as changes in nutritional status first affect ghrelin secretion and after that they affect the expression or the circulating ghrelin levels. Moreover, it has been shown that exposure to food-related sensory stimuli without subsequent food intake is able to modify gastric ghrelin secretion and its circulating levels in the same way as true feeding does [17]. This proves that other factors apart from those produced by nutrient ingestion or absorption are involved in the regulation of this hormone [20]. In addition, we found that the regulation of ghrelin secretion from the stomach by food-related stimuli is mediated by a medium-term memory mechanism from the sensory neural system, which is integrated into the enteric nervous system. The vagus nerve is thought to mediate the effects of ghrelin, as the regulation of gastric ghrelin secretion by sensory stimuli is blocked after surgical vagotomy in rats [17].

Age and Sex Regulate Ghrelin Secretion and GOAT mRNA Levels from Isolated Rat Stomach

It was shown that plasma ghrelin levels are not affected in a significant way by age; however, by using the organ explants system it was found that ghrelin secretion from the stomach is altered in female animals at the beginning of the pubertal stage coinciding with an increase in the uterus weight [21]. Puberty is a stage of life

characterized by strong modifications in circulating levels of estrogen and testosterone. Under this context, the theory of a possible direct effect of estrogens on the stomach to regulate ghrelin secretion emerged and was subsequently proven by applying the culture system of gastric tissue explants to ex vivo and in vitro studies. A direct effect of estrogen treatment on gastric tissue was observed by inducing a significant decrease in ghrelin secretion with no effect on gastric mRNA and ghrelin protein levels [21]. In contrast, in the males, puberty starts at 6 weeks of age, as shown by the increase in testosterone levels, coinciding with the highest levels of gastric ghrelin secretion. However, it was found that direct treatment with testosterone on the stomach did not affect gastric ghrelin secretion in this model. In the same article the effect of estrogens and testosterone was tested ex vivo, reproducing in the lab the physiological regulation of gastric ghrelin secretion by estrogens that occur in the puberty. The increased levels of estrogen, characteristics of the puberty in rat, were prevented after surgical ovariectomy; this prevented the decrease in gastric ghrelin secretion found at puberty in female rats. Furthermore, when estrogen levels were replaced by exogenous estradiol treatment in ovariectomized animals, ghrelin secretion was normalized [21].

It is widely known that puberty is a period of development with high energetic requirements associated with the growing process. In addition the study showed that it is during puberty that the levels of GOAT expression in the stomach are the highest [21]. The increased gastric GOAT expression during periods of increased energy requirements suggests a role for GOAT as a part of the mechanism for maintaining energy balance as it allows the organism to conserve energy and to counteract states of negative energy balance.

Lactating Status Regulates Gastric Ghrelin Secretion

A relevant period of life is weaning, as it is a process associated with modifications in gastrointestinal tract morphology, as well as dietary and environmental changes [22]. Weaning represents a step from breastfeeding to solid intake and in the rat takes place immediately prior to the 4th week of life. Gastric ghrelin secretion, as measured with the organ explant system, was found to be elevated during the breastfeeding period (1–3 weeks of life) and to decline after weaning (4 weeks of life) [21]. It is possible that the gastric ghrelin secretion modifications found at 4 weeks of life might be produced as a consequence of the effects elicited by weaning on the maturation of ghrelin-producing cells and morphology in the gastrointestinal tract [23]. To test this hypothesis the direct gastric ghrelin secretion was measured with the explants model in animals subjected to a delay in weaning. In the female pups, the delay of weaning did not affect gastric ghrelin secretion probably due to the powerful effect of estrogen variations produced at this age and described above, which may be masking a possible effect. However, in 4-week-old male animals the delay of weaning prevented the drop in gastric ghrelin secretion found in weaned animals [21]. On the other hand, delays in weaning decrease testosterone levels in

rats, as well as GOAT mRNA levels [21]. The study further explored this finding by assaying the variations in GOAT expression by the stomach under variations of testosterone levels both in vivo and in vitro and found that testosterone regulates GOAT expression [21].

Macronutrients Act Directly on the Stomach to Regulate Gastric Ghrelin Release

The composition of the diet has been revealed as a factor regulating gastric ghrelin secretion, as the satiating capacity depends on the nature of the different macronutrients in the food. Further, the composition of the ingested food has been shown to affect digestive and secretory process in the gastrointestinal tract. Recently, T1R taste receptors able to detect macronutrients, which are normally present in the gustatory system, were identified in the proximal intestinal mucosa [24, 25]. The expression of the T1R3 subtype, which is essential for the detection of both sugars and amino acids (aa) [26], was detected in two distinct cell populations of the gastric mucosa [27]. More specifically, these receptors have been identified in the open-type brush cells, which are in contact with the luminal content, and the closed-type brush cells, both of which produce ghrelin. These findings suggest a possible interaction between the gastric chemosensory system and ghrelin secretion regulation. This theory is supported by the fact that ghrelin regulation depends on nutritional status and, in particular, the type of macronutrient ingested. It has been shown that carbohydrates cause a more potent inhibitory effect than fat on gastric ghrelin content and on plasma ghrelin levels [28, 29]. A recent study using the gastric explant model was the first to show that macronutrients—specifically, amino acids and lipids—act directly on the ghrelin-producing cells of the stomach to regulate gastric ghrelin release [30].

Regulation of Gastric Ghrelin Secretion by Components of the Somatotrope Axis

In addition to factors related to food intake, other hormonal factors are implicated in gastric ghrelin regulation. Other metabolic stimuli, such as the classical components of the somatotrope axis, are able to regulate ghrelin secretion. Among these factors are included somatostatin (SS), growth hormone-releasing hormone (GHRH), insulin-like growth factor 1 (IGF-1), and growth hormone (GH) [31]. In a study using the organ culture model of gastric tissue capable of assessing the direct regulation of ghrelin secretion by the stomach [17], the role of the components of the somatotrope axis on gastric ghrelin secretion was tested. Two to three hours of incubation with SS significantly decreased ghrelin secretion directly from the stomach

in vitro, suggesting that plasma ghrelin changes induced by these hormones would be directly due to changes in gastric ghrelin release. Supporting this study, some SS-producing cells make direct cellular contact with ghrelin-producing cells in the gastric fundus [32, 33]. In addition, SS receptor is expressed in the gastric mucosa [34]. Under this context, the reduction in plasma ghrelin levels by SS [35] is mediated by a direct inhibitory action of SS on the stomach. Negative GH feedback on stomach ghrelin has been suggested [36]. In addition GH receptors are present in the stomach and the intestine [37]. By using the organ explants model the first evidence for a direct GH effect on gastric ghrelin secretion was proposed based on the fact that GH treatment directly on gastric tissue explants induced an inhibitory effect on ghrelin release from the stomach [31]. In contrast, the authors did not find both GHRH and IGF-1 effects on basal gastric ghrelin secretion.

In summary, the stomach has its own mechanism for ghrelin/GOAT regulation to maintain energy balance, allowing the organism to adapt to the different needs of each period of life by switching the ratio of acyl/desacyl ghrelin. This mechanism acts as a gastric medium-term memory system and interacts with the CNS through the vagus nerve to transmit the information to the primary brain centers involved in energy balance regulation. In addition, this mechanism of gastric ghrelin regulation is influenced by nutritional status, different hormonal factors, age, sex, and lactating status [17, 21].

Ghrelin Actions

After more than 11 years of research on this topic, ghrelin is now known to be a pleiotropic hormone and, as a result, displays a wide spectrum of biological functions, such as the regulation of appetite and food intake, gastric motility, gastric acid secretion, endocrine and exocrine pancreatic secretions, cell proliferation, glucose and lipid metabolism, and cardiovascular and immunologic processes [38]. Of the neuroendocrine functions of ghrelin, the most notable is the remarkable effect of the exogenous administration of the peptide on GH secretion, as well as its impact on the corticotroph axis and prolactin secretion [39]. In this context, defective ghrelin signaling from the stomach could contribute to abnormalities in energy balance, growth, and associated gastrointestinal and neuroendocrine functions.

Ghrelin Regulation of Energy Balance

Gut–brain communication is exerted by a multifactorial, complex mechanism composed of afferent stimuli from the gastrointestinal tract (e.g., nutritional, nervous, and metabolic stimuli) and efferent orders from the SNC responding to peripheral signals. The maintenance of energy homeostasis is dependent on the balance between caloric intake and energy expenditure and requires coordination

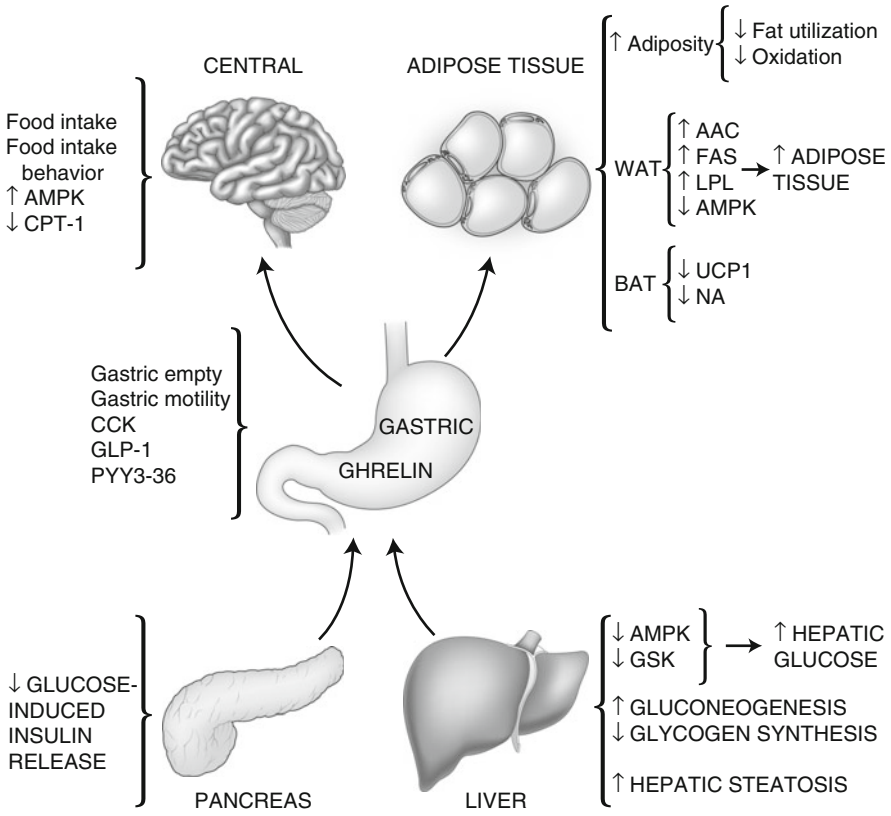

Fig. 4.1 Gastric ghrelin regulates energy balance acting at different levels and through different mechanisms. It regulates food intake and food intake behavior in the brain by actions at both hypothalamic and extra-hypothalamic centers. In adipose tissue ghrelin induces adiposity by a reduction of fat utilization and oxygen consumption. The regulation in lipid storage in white adipose tissue (WAT) is mediated by its effects on lipid metabolism pathway. In brown adipose tissue (BAT) the sympathetic nervous system enervating adipose tissue is a target for ghrelin action where it inhibits noradrenaline (NA) release. In addition chronic ghrelin induces a decrease of UCP-1 in BAT. At hepatic level, ghrelin affects glucose metabolism inducing an increase in hepatic glucose as a consequence of an increased gluconeogenesis and decreased glycogen synthesis by an inhibition of protein kinase AKT and glycogen synthase kinase (GSK) activation. Lipid metabolism in the liver is also affected by ghrelin; it produces hepatic steatosis. In the pancreas ghrelin suppresses glucose-induced insulin release. Finally, ghrelin's interaction with other peptides from the gastrointestinal tract (CCK, GLP-1, and PYY3-36) is implicated in energy balance regulation. In addition, ghrelin's modification of gastric emptying and motility can mediate the effects on food intake

between peripheral signals and central regulatory networks in charge of body weight maintenance [40].

Gastric ghrelin acts at different levels and through different mechanisms to regulate energy balance (Fig. 4.1). For example:

– Ghrelin regulates food intake at a central level. This occurs, in part, via the action of ghrelin on hypothalamic centers implicated in appetite control and, in part, by its actions on other extra-hypothalamic brain areas involved in feeding behavior.
– Ghrelin acts on body weight regulation through its actions on metabolism and energy expenditure, i.e., ghrelin induces adiposity by a reduction in fat utilization and oxygen consumption. The energy expenditure is controlled via the sympathetic nervous system, which innervates the adipose tissue. In addition, effects of ghrelin have been described on glucose homeostasis.
– Ghrelin's interactions with other peptides from the gastrointestinal tract are responsible for energy balance regulation. Intestinal function may be related to energy balance. Gastric distension acts as a satiety signal to inhibit food intake and rapid gastric emptying is related to overeating and obesity.

Central Ghrelin Regulation of Food Intake

Mechanism of the Orexigenic Effect of Ghrelin in the Hypothalamus

Ghrelin's impact on feeding is thought to occur through its ability to influence activity in the hypothalamic centers involved in metabolic homeostasis (Fig. 4.2).

Peripheral signals implicated in regulation of energy balance act directly at hypothalamic neuronal pathways or indirectly through additional pathways. Hypothalamic nuclei respond to changes in energy status by modifying the expression of specific neuropeptides, which cause changes in energy intake and expenditure [41].

The orexigenic effect of ghrelin is exerted through the GHS-R, which is highly expressed in the hypothalamus and mainly in the arcuate nucleus [42]. In the arcuate nucleus the NPY/AgRP neurons are the primary targets of the orexigenic actions of ghrelin [43]. Ghrelin treatment to both ad libitum fed and fasted rats led to a clear increase in NPY/AgRP mRNA content in the ARC [44]. Moreover, peripheral administration of ghrelin increased c-fos expression in ARC NPY/AgRP neurons, and ablation of these neurons completely abolished the orexigenic effect of ghrelin [18].

In the last decade, numerous studies have tried to identify the mechanism behind the orexigenic effect of ghrelin induced by a modulation of NPY/AgRP in the hypothalamus. The role of AMP-activated protein kinase (AMPK) in ghrelin-induced hypothalamic appetite regulation has recently been demonstrated. Ghrelin induces changes in the AMPK-CPT1 pathway that are associated with increased levels of AgRP and NPY mRNA expression in the ARC [45]. In addition, it was recently found that ghrelin also decreases the expression of fatty acid synthase (FAS), specifically in the ventromedial nucleus of the hypothalamus (VMH), which may contribute to the peptide's orexigenic effect [46].

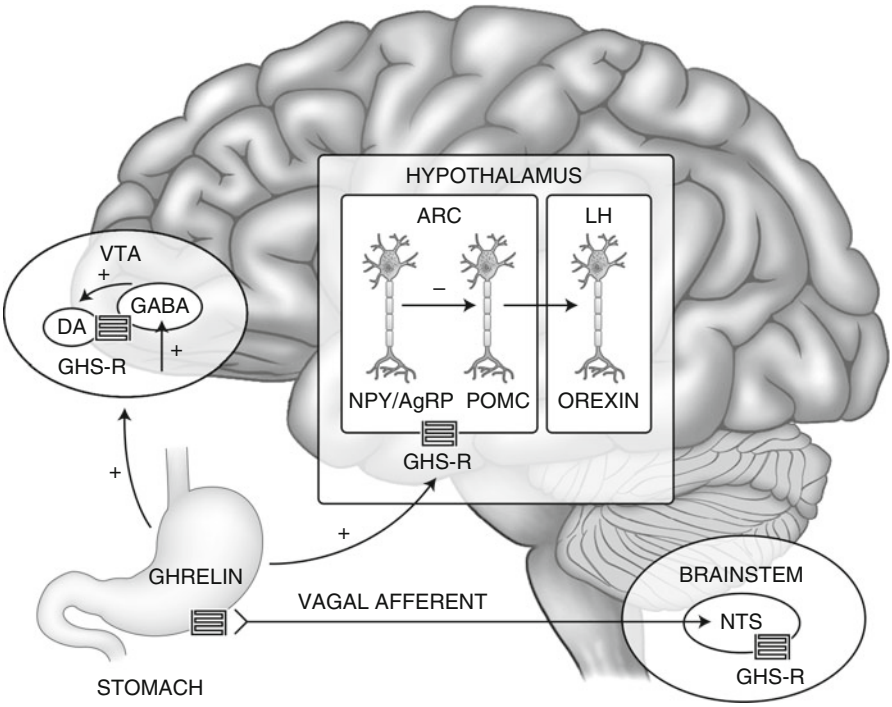

Fig. 4.2 Ghrelin induces food intake by activation of NPY/AgRP hypothalamic neurons in the arcuate nucleus (ARC) and indirectly inhibiting POMC neurons and Orexin neurons in the lateral hypothalamus (LH). In addition other extra-hypothalamic areas are implicated in ghrelin's orexigenic effects including the dorsovagal complex in the brainstem. At this level, the nucleus of tractus solitary (NTS) receives the vagal afferent signals from the stomach. Another central target for ghrelin action is the ventral tegmental area (VTA) of the brain projecting to forebrain regions implicated in dopamine (DA) release in response to or anticipation of reward. VTA dopamine cells express ghrelin receptor (GHS-R) and ghrelin-induced DA release from these cells is probably mediated by glutamatergic neurotransmission. The possibility of a vagal efferent-mediated action of peripheral ghrelin on VTA has been proposed

Bsx (the hypothalamic homeo box domain transcription factor) was thought to be involved in the generation of hyperphagic responses in the central nervous system [47]. Ghrelin regulates Bsx expression in the ARC, suggesting that this transcription factor could be mediating the effects of ghrelin on NPY/AgRp neurons [47]. Bsx is also known to interact with other transcription factors: the forkhead Fox O1 (FoxO1) and the phosphorylated cAMP response-element-binding protein (pCREB) to modulate the expression of AgRP and NPY, respectively [48].

Besides these effects in the arcuate nucleus, ghrelin inhibits ARC POMC neurons by antagonizing MC4R-containing neurons in the PVN [49].

In summary, ghrelin induces central orexigenic effect by the modulation of NPY/AgRP in the hypothalamus. This action is mediated by the activation of fatty acid

oxidation pathways and reactive oxygen species buffering in NPY/AGRP neurons, which has been revealed to be dependent on the transcription factors Bsx, Fox O1, and pCREB [45].

Other Extrahypothalamic Areas Involved in Ghrelin Effects

At the CNS, GHS-R expression is not limited to the hypothalamus, and other structures relevant to feeding control may also contribute to physiological actions of ghrelin. Indeed, the administration of ghrelin in different extrahypothalamic areas of the brain induces positive energy balance [50].

The dorsal vagal complex (DVC) localized in the brainstem is a key center relevant in the neural control of feeding behavior [51]. It is responsible for coordinating peripheral signals, such as the vagal afferents connecting the stomach and hypothalamus. Vagus afferent signals communicate events that take place at the gastric level to the CNS, and vagal afferent neurons express ghrelin in their terminations in the brainstem. GHS-R is expressed in the DVC, and a hyperphagic response has been observed after the microinjection of ghrelin into this complex [50]. Ghrelin injection increases c-fos activity in the brainstem, particularly in the nucleus tractus solitary (NTS) and area postrema (AP). The blockade of the gastric afferent vagal nerve in rats abolishes ghrelin-induced feeding and prevents the ghrelin-induced rise in c-fos expression in the arcuate, supporting the role of the vagus nerve in mediating the orexigenic actions of gastric ghrelin [52].

Additionally, the subfornical organ (SFO) constitutes a CNS structure at the blood–brain interface. It has been shown that efferent projections of SFO to hypothalamic autonomic control centers modify energy balance [53]. SFO expresses the ghrelin receptor [54], and whole-cell patch recordings have shown that ghrelin has an excitatory effect on SFO neurons after administration of a minimal effective concentration [54]. Thus, SFO neurons might be additional central targets for ghrelin to induce its orexigenic effect.

On the other hand, the mesolimbic dopaminergic system consists of a subpopulation of dopamine neurons in the ventral tegmental area (VTA) that project to forebrain regions, where they release dopamine in response to, or in anticipation of, rewards [55]. This pathway is thought to have evolved into a system directly involved in primary behaviors related to motivational and rewarding aspects of food-seeking [56]. A total of 50–60% of VTA dopamine cells express GHS-R, indicating that ghrelin can potentially have a large influence on the release of dopamine from these cells [42]. The infusion of ghrelin into the VTA increases food intake dramatically, inducing dopamine release via an increase in dopamine cell excitability. In addition, this effect appears to be mediated by increased glutamatergic neurotransmission [57]. Given the fact that VTA is a brain area protected by the BBB, it is possible that the effects of ghrelin on the activity and neurotransmitter

release of VTA dopamine neurons may be produced, at least in part, indirectly though the actions of ghrelin on afferent systems connected to the VTA [58]. The VTA is heavily innervated by lateral hypothalamic neurons that produce orexin, constituting possible candidates for the attenuation of ghrelin's effect on food intake [58] (Fig. 4.2).

Peripheral Ghrelin Regulation of Energy Balance

The Actions of Ghrelin on Adipose Tissue and Liver Are Mediated by Affecting Lipid Metabolism

Chronic ghrelin administration induces adiposity in rodents and humans [18]. Biochemical and morphological analysis of adipose tissue revealed that ghrelin treatment induced an increase in the triglyceride content of white adipose tissue (WAT) and a corresponding increase in cell size of adipocytes [59].

The role for ghrelin in the regulation of WAT is thought to be mediated by its effect on lipogenesis which is produced by an increase in lipogenic enzyme expression, fat accumulation, and an inhibition of lipid oxidation in white adipocytes [60]. It has been shown that central ghrelin administration increases the expression of acetyl-coA carboxylase (ACC), FAS, lipoprotein lipase (LPL), and fat oxidation-promoting carnitoyl-palmitoyl transferase 1 (CPT-1) in WAT. The effects of ghrelin on the lipid metabolism pathway in the adipocyte are opposite to those described in the hypothalamus (see section above). In line with this, it has been shown that the direct peripheral effect of ghrelin on adipose tissue involves the inhibition of AMPK activity at this level, which provokes the increase in adipose tissue mass [61] (Fig. 4.1).

On the other hand, an additional mechanism mediating the effect of ghrelin on energy balance regulation involves the modulation of energy expenditure by its action on brown adipose tissue (BAT). BAT plays a major role in energy expenditure and thermogenesis via the function of uncoupling protein 1 (UCP1). UCP-1 mRNA was found to be decreased in BAT in response to chronic repeated treatment with ghrelin [59]. However, there were no changes in UCP-1 in response to acute central/peripheral ghrelin administration (Fig. 4.1) [62].

The regulation of energy expenditure in BAT is under the control of the sympathetic nervous system and higher brain structures [63]. Moreover, it has been shown that ghrelin administered centrally or peripherally inhibits noradrenaline release in BAT in rats. In addition, it has been shown that GHS-R1a expressed in the vagal nerve may be involved in the signal transduction of peripheral ghrelin to control the function of brown adipocytes [62].

At the hepatic level, ghrelin administration induces hepatic steatosis, an increased number of lipid droplets, and triacylglycerol content via its interaction with the GHS-R (Fig. 4.1) [60, 64]. De novo lipogenesis in the liver induced by ghrelin occurs in a GH-independent fashion; however, lipid mobilization occurs in a GH-dependent fashion [65].

In summary, ghrelin affects lipid metabolism in adipose tissue and the liver, thus contributing to the storage of fat.

The Actions of Ghrelin in Glucose Metabolism and Insulin Sensitivity

The acute administration of ghrelin induces hyperglycemia and reduces insulin secretion in healthy humans [66], but not in obese patients [67]. In addition, plasma ghrelin levels are shown to correlate negatively with insulin concentrations and are associated with elevated fasting insulin levels, insulin resistance, and obesity [68, 69].

Differential actions of ghrelin on glucose homeostasis have been found between acyl-ghrelin (AG) and des-acylghrelin (UAG). On the one hand, AG has been shown to decrease insulin and raise glucose levels, while no effects were found with UAG. Despite the lack of effect of UAG on glucose metabolism when administered alone, the unacylated form is able to counteract the effects of AG on insulin secretion; however, it does not act as a stimulator of GH, prolactin, ACTH, and cortisol levels [66]. Moreover, the combination of AG and UAG improves insulin sensitivity [66]. Several data have suggested that ghrelin is produced in the pancreas where it might be involved in the regulation of insulin production [70, 71]. Accordingly, it has been shown that UAG stimulates insulin production in pancreatic cell lines [72]. In addition, it has been shown that in anesthetized rats, UAG induces insulin secretion in the portal blood in a manner opposite to that seen in the circulating blood, indicating a direct effect of UAG on liver glucose metabolism [72].

Several pharmacologic studies have shown that the use of antagonist of GHSR could improve glucose tolerance and insulin resistance in the long term, which suggest that this compound could be a future therapy for the treatment of type 2 diabetes [73–76].

The mechanism behind the increase in hepatic glucose production induced by ghrelin involves a hepatic decrease in protein kinase AKT and glycogen synthase kinase (GSK) activation, both of which are protein kinases downstream of the insulin receptor is involved in the suppression of hepatic gluconeogenesis. The changes induced by ghrelin administration are associated with enhanced peroxisome proliferator-activated receptor-γ-coactivator (PGC-1α), which activates gluconeogenesis in the liver (Fig. 4.1) [77].

A key pathway in metabolism regulation is the insulin stimulation of glucose uptake in adipose and muscle tissue. An essential event for the uptake of glucose

by fat is the translocation of the glucose transporter, GLUT-4, to the plasma membrane from intracellular storage sites [78]. It was shown as ghrelin modulates glucose transport in adipose tissue probably mediated by actions on the lipid kinase phosphatidylinositol PI3K/AKT pathway (Fig. 4.1) [79].

In summary, ghrelin regulates glucose metabolism at three different levels. In the liver by upregulating hepatic gluconeogenesis and downregulating glycogen synthesis; in the pancreas, ghrelin acts by suppressing glucose-induced insulin release and finally, in adipose tissue, it works by increasing glucose transport into the adipocytes (Fig. 4.1).

Gastric Actions of Ghrelin Regulating Energy Balance by the Interaction with Gastrointestinal Hormones Signaling Satiety

Different peptides in the gastrointestinal tract may inhibit food intake, in part, by inhibiting gastric emptying. In contrast, ghrelin has been shown to stimulate both gastric emptying and food intake, possibly through its interaction with the following peptides (Fig. 4.1). As an example, CCK is a hormone with anorexigenic properties that has been shown to reduce size and duration of the meal in all species studied [80]. CCK inhibits gastric emptying through its action on CCK1-specific receptors located in the gastric antrum [81]. It has been suggested that the orexigenic effect of ghrelin is blocked after the peripheral administration of CCK-8 and that this effect may be mediated by vagal afferent function [82]. Other gastrointestinal hormone, GLP-1, is postprandially released primarily from the distal small intestine and colon L cells [83]. In humans, GLP-1 reduces energy intake, gastric emptying rates, and energy consumption [84]. It has been suggested ghrelin and GLP-1 antagonize one another in the regulation of food intake [85]. In addition, an inverse relationship between circulating levels of ghrelin and GLP-1 has been described [86], which may indicate an interaction between these two peptides.

One study provided evidence that ghrelin may stimulate food intake, in part, by attenuating the inhibitory effects of GLP-1 and PYY 3–36 on gastric emptying and food intake [85].

Conclusions

In summary, the stomach itself regulates ghrelin and GOAT production to increase the ability of the organism to adapt to the varying metabolic requirements required through each stage of life. In addition, gastric ghrelin secretion is regulated depending on the nutritional status which in turn acts by informing the central pathways controlling energy balance of the availability of substrates within the organism. The mechanisms by which ghrelin regulates appetite and metabolism are mediated via a complex system involving central and peripheral elements. The peptide induces

adiposity by stimulating food intake and promoting fat storage, and it also reduces energy expenditure and increases glucose uptake in adipose tissue.

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Chapter 5
Ghrelin Regulation of AMPK in the Hypothalamus and Peripheral Tissues

Miski Scerif, Blerina Kola, and Márta Korbonits

Abstract Adenosine 5′ monophosphate-activated protein kinase (AMPK) is a regulator of cellular and systemic energy homeostasis. Several appetite-regulating and metabolic hormones are known to influence AMPK. Ghrelin displays central orexigenic as well as direct peripheral metabolic effects and both could be related to its effects on AMPK activity. In the hypothalamus ghrelin stimulates AMPK and this has been associated with its orexigenic effects. Evidence suggests a tight interaction between the central ghrelin and cannabinoid systems, and hypothalamic AMPK appears to be a common mediator of their orexigenic effects. In the liver and adipose tissue, ghrelin inhibits AMPK and this could be the underlying mechanism for its diabetogenic and lipogenic effects. While in cardiac muscle, ghrelin stimulates AMPK which could be linked to its positive inotropic effects. In summary, tissue-specific alterations of AMPK activity play an important role in the diverse effects of ghrelin.

M. Scerif, M.B.B.S., B.Med.Sci. • B. Kola, M.D., Ph.D.
• M. Korbonits, M.D., Ph.D. (✉)
Centre for Endocrinology, William Harvey Research Institute,
Barts and The London, Queen Mary's School of Medicine and Dentisty,
Charterhouse Square, London EC1M 6BQ, UK
e-mail: m.kornonits@qmul.ac.uk

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Introduction

AMP-Activated Protein Kinase

AMPK: Structure and Function

Adenosine 5′ monophosphate-activated protein kinase (AMPK) is a key regulator of cellular and systemic energy homeostasis and acts as a sensor of energy status. AMPK was first named in 1988; however, it appeared in several earlier reports under the names of HMG-CoA reductase kinase and acetyl-CoA carboxylase kinase [1]. AMPK is an evolutionally well-conserved heterotrimeric serine/threonine kinase composed of a catalytic α (alpha) subunit and regulatory β (beta) and γ (gamma) subunits [2]. The α subunit contains the kinase domain and an autoinhibitory region. The β subunit contains a glycogen-binding domain, provides the scaffold for the complex, and has an autoinhibitory function. The γ subunit comprises four cystathionine-β-synthase (CBS) motifs [3]. At the interface of the CBS motifs there are four potential AMP binding sites. Two of these binding sites can bind either AMP or ATP, whilst the third site contains a tightly bound AMP that does not exchange (Fig. 5.1). The fourth potential binding site shows a single difference in amino acid structure and this probably accounts for the fact that AMP does not bind

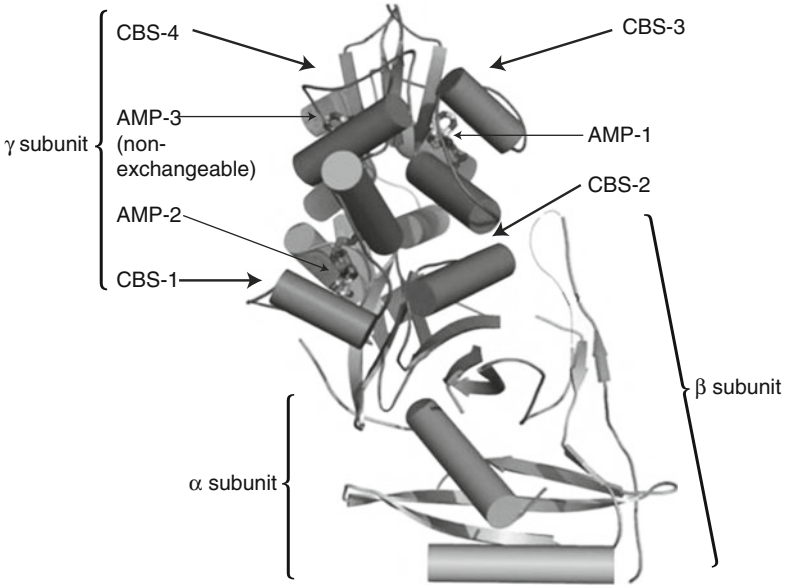

Fig. 5.1 Structure of AMPK. Ribbon representation of the crystallised complex with bound three AMP molecules. *AMP* adenosine monophosphate, *CBS* cystathionine-β-synthase motif. Adapted from [3]

Table 5.1 Human chromosome locations of genes encoding AMPK subunits

Subunit	Isoform	Chromosome location of gene
Alpha	α1	5p13.1
	α2	1p32.2
Beta	β1	12q24.23
	β2	1q21.1
Gamma	γ1	12q13.12
	γ2	7q36.1
	γ3	2q35

at this site. When AMPK is inactive it binds two ATP and one AMP molecules; however, in low-energy states it binds three AMP molecules [3].

The α and β subunits each display two isoforms, whilst the γ subunit has three isoforms; all isoforms are the product of different genes (Table 5.1). The assembly of these subunits can theoretically lead to 12 possible αβγ combinations [4]. The AMPK protein is expressed in all mammalian tissues and different isoform combinations differ in their subcellular localisation and tissue distribution [5]. The liver expresses both α1 and α2 isoforms, whilst in adipose tissue the α1 subunit is the predominant catalytic isoform and accounts for a major part of AMPK activity [6].

AMPK may be activated through physiological and pathological mechanisms [2]. Inhibition of ATP synthesis by pathological processes such as glucose deprivation, ischaemia, hypoxia, heat shock, metabolic poisons and oxidative stresses [4] leads to AMPK activation. Physiologically, muscle contraction accelerates ATP consumption, and thus activates AMPK [5]. In summary, AMP:ATP ratio is raised by increased ATP consumption or reduced ATP production and this leads to AMPK activation. AMP binding leads to conformational change and activation of AMPK [3]. AMP had been suggested to activate AMPK in three distinct ways: (1) allosterically, (2) by stimulating phosphorylation by upstream kinases and (3) by inhibiting dephosphorylation by protein phosphatases [4, 7, 8]. However, recent data suggest that increased AMP does not directly promote phosphorylation of AMPK by upstream kinases but rather inhibits its dephosphorylation by protein phosphatase-2Cα (PP2C-α) [8]. Although AMPK received its name because of the allosteric activation by AMP, this effect is rather small compared with the effect of phosphorylation by upstream kinases [5].

AMPK upstream kinases include LKB1 (a tumour-suppressor gene), calmodulin kinase kinase 1 and 2 (CaMKK1 and 2, also known as CaMKKα and β, which confer the effect of increased cytoplasmic calcium levels), transforming growth factor-β-activated kinase 1 (TAK1) [7], kinase suppressor of Ras 2 (KSR2) [9] and other AMPK kinases that continue to be discovered [10]. These upstream kinases activate AMPK by phosphorylating a specific threonine residue (Thr172) within the activation loop of the kinase domain on the α subunit [5]. AMPK can also be regulated directly by glycogen through allosteric inhibition, and may act as a glycogen sensor. In addition to the allosteric effect, the branched glycogen molecule also inhibits LBK1- and CaMKK2-induced Thr172 phosphorylation [7].

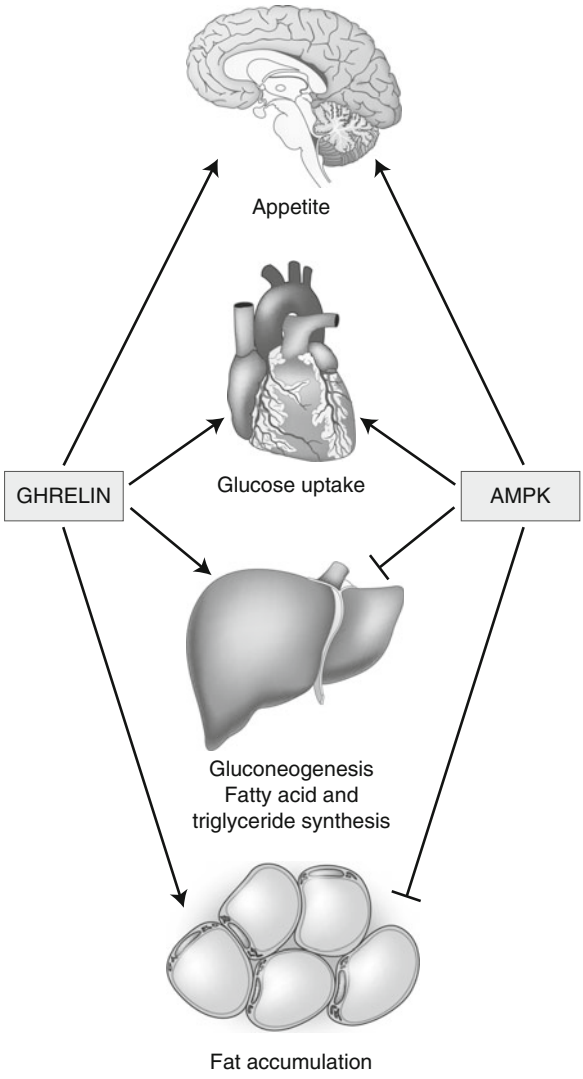

Fig. 5.2 Comparison of prominent effects of AMPK and ghrelin in various tissues. → stimulation; ⊣ inhibition. Adapted from [4]

Once activated, AMPK leads to a switch from anabolic to catabolic pathways. With the aim of restoring energy balance, ATP production is increased and energy-utilising pathways are inhibited. AMPK can have acute effects through direct phosphorylation of target enzymes and long-term effects through phosphorylation of transcription factors and histones [4, 11]. Activation of hypothalamic AMPK leads to an increase in appetite [12]. AMPK activation is protective in myocardial ishaemia. AMPK activation in the liver leads to inhibition of gluconeogenesis and inhibition of fatty acid and cholesterol synthesis. In adipose tissue, activation of AMPK leads to reduced lipid stores with inhibition of lipogenesis but also reduced lipolysis with reduction in free fatty acid release (Fig. 5.2).

AMPK and the Endocrine System

AMPK has been described as the mediator of the action of several hormones [4]. Leptin [12, 13], adiponectin [14–18], insulin [12, 19, 20], glucocorticoids [21–23] and ghrelin [24] interact with AMPK, which is involved in their metabolic effects. This key regulator of energy homeostasis has also been implicated in mediating the metabolic effects of cannabinoids [7]. In addition, AMPK has been suggested to mediate the action of metformin [25] via LKB1 [26]. However, as metformin inhibits complex type I of the mitochondrial respiratory chain [27], it has also been suggested that the AMPK stimulatory effect of metformin is indirect as it leads to leaky mitochondria and reduced ATP synthesis. A recent study, using double α1–α2 liver KO mice, suggested that AMPK is not needed for the effects of metformin [28]. The LKB1/AMPK pathway has been reported to regulate the phosphorylation and nuclear exclusion of CREB-regulated transcription co-activator 2 (CRTC2) [26]. CRTC2, also referred to as TORC2, is a transcriptional co-activator that mediates CREB-dependent transcription of PGC-1α (PPARγ co-activator-1α) and its gluconeogenic target genes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [29]. However, recent findings indicate that the regulation of gluconeogenic gene expression by metformin is dependent on the phosphorylation of CREB-binding protein (CBP), but not CRTC2 [30].

It remains to be clarified why some hormones and compounds, including leptin, ghrelin and cannabinoids, have opposite effects on AMPK in different tissues. This might be the result of different patterns of AMPK subunit expression in the various tissues, or might be due to the differential expression of the AMPK activators, LKB1, CaMKK2 or others [4], and/or AMPK inhibitors.

Cannabinoids

Research into the chemical constituents of the cannabis plant and their mechanism of action led to the identification of the main psychoactive component, Δ^9-tetrahydrocannabinol (THC) and subsequently the discovery of its cellular $G_{i/o}$ protein-coupled receptors, cannabinoid receptor type 1 and 2 (CB1 and CB2) and their endogenous ligands, the endocannabinoids. CB1 is considered one of the most prevalent receptors in the mammalian brain, with anandamide (AEA) and 2-arachidonoylglycerol (2-AG) being the most widely studied endocannabinoid ligands [31]. Unlike other neurotransmitters, the endocannabinoids are not stored in vesicles but produced on demand by Ca^{2+}-induced enzymatic cleavage from phospholipid precursors and then rapidly degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol (MAG) lipase [32]. CB1 receptors are often localised on presynaptic neurons, which suggests retrograde signal transmission [33], whereby the endocannabinoids act to reduce neurotransmission of the pre-synaptic neuron via inhibitory effects on voltage-gated Ca^{2+} channels and the activation of K^+ channels, or by reduced cAMP generation [34].

The endocannabinoids have effects on various systems including the immune, gastrointestinal, cardiovascular and reproductive systems. There are also effects on mood, cognition and reward, and neuroprotective functions. In addition, endocannabinoids stimulate appetite and have effects on lipid and carbohydrate metabolism [33]. Hypothalamic endocannabinoid levels have been shown to increase with fasting and to decrease after re-feeding, indicating a role in appetite control [35]. Interestingly, hypothalamic cannabinoids have been shown to be negatively regulated by leptin, playing therefore a role in its anorexigenic effect [36]. CB1-immunoreactive axons densely innervate all feeding-related nuclei in the hypothalamus, including the arcuate (ARC) and the paraventricular (PVN) nuclei, via both excitatory and inhibitory synapses [37]. The endogenous cannabinoids AEA and 2-AG increase appetite specifically via the CB1 receptor [33, 35, 38]. This was further confirmed by a study that showed both exogenous and endogenous endocannabinoids to induce food intake via the CB1 receptor [39]. More recently, the peptide hemopressin which acts as a reverse agonist at the CB1 receptor has been shown to reduce food intake in rats and mice [40].

Both animal and human data show that the endocannabinoid system is up-regulated in obesity [41]. Tam et al. have recently shown that AM6545, a non-brain-penetrant neutral CB1 receptor antagonist, does not affect behavioural responses mediated by CB1 receptor in the brains of mice with genetic or diet-induced obesity, but it does cause weight-independent improvements in glucose homeostasis, fatty liver, and plasma lipid profile [42]. Whilst neuropsychiatric side effects of central CB1 receptor blockade have led to the demise of rimonabant in therapeutic medicine, there are now suggestions that selective targeting of peripheral CB1 receptors has therapeutic potential for alleviating cardiometabolic risk in obese patients [42].

AMPK has been found to be a common mediator of the central orexigenic and peripheral metabolic effects of ghrelin and cannabinoids. It seems that cannabinoids interact with a number of hormonal systems and perhaps mediate their effects [31]. There are data for heterodimerisation of the cannabinoid receptor with other G-protein-coupled receptors (GPCR) [43–45], and the ghrelin receptor (GHS-R) has been suggested as a possible partner [46]. We have shown that the orexigenic effect of ghrelin is blocked in the presence of a cannabinoid receptor type 1 (CB1) antagonist [47]. This suggests a functional relationship between ghrelin and the cannabinoid system in the hypothalamus, and raises the possibility that the effect of ghrelin on food intake is mediated through stimulation of cannabinoid release.

Ghrelin Regulation of AMPK in the Hypothalamus

Ghrelin is one of the most powerful orexigenic peptides. Most orexigenic peptides originate from the brain and are active only when injected into the brain, while ghrelin is active even with peripheral administration leading to an increase in appetite in rodents and humans [48–50]. Ghrelin activates neuropeptide Y

(NPY)/agouti-related peptide (AgRP) neurons [51] of the hypothalamic ARC through its GHS-R1a receptor, promoting production and secretion of their orexigenic neuropeptides, which suppress pro-opiomelanocortin (POMC) neuronal activity while promoting food intake [52, 53]. When GHS-R receptor is diminished in the ARC rats exhibit reduced food intake and decreased body weight [54] and lack of GHS-R protects mice from obesity under chronic exposure to high-fat diet [55]. Mice with double knockout (KO) of NPY/AgRP genes completely lack the orexigenic action of ghrelin, confirming that these neuropeptides mediate and are essential for the orexigenic effect of this peptide hormone [56]. NPY/AgRP neurons are essential for feeding and their ablation in adulthood leads to starvation [57].

Hypothalamic AMPK has been identified to play a vital role in the regulation of food intake. It has recently been shown that AMPK β1 deletion in mice reduces appetite and protects from diet-induced obesity [58]. Dominant negative AMPK expression in the hypothalamus is sufficient to reduce food intake and body weight; whereas constitutively active AMPK increases both food intake and body weight [12]. Evidence that AMPK activity in the hypothalamus is regulated by hormones that have counter-regulatory and opposing effects on food intake such as leptin and ghrelin [12, 59] further demonstrates that AMPK is directly involved in appetite regulation. Ghrelin is functionally a natural antagonist to the anorexic hormone leptin [48] and while leptin inhibits hypothalamic AMPK activity [12, 59], ghrelin has an opposing stimulatory effect [24, 59–61]. Lerner et al. propose a role for the transcriptional co-activator CRTC2 in hypothalamic mechanisms linking glucose sensing with AMPK and gene regulation. They suggest that low glucose and fasting states are associated with hypothalamic AMPK activation, leading to phosphorylation and nuclear exclusion of CRTC2, and thus reduced transcription of cAMP response element (CRE) genes, insulin receptor substrate 2 and anorectic corticotropin-releasing hormone (CRH) [62]. Ghrelin stimulates CRH [63] and this is consistent with its ACTH-releasing effect. However, the effect of ghrelin on appetite may occur via a different pool of CRH neurons [64].

AMPK has been proposed to mediate the orexigenic effect of ghrelin [24, 59–61]. Our work has demonstrated the stimulatory effect of ghrelin on hypothalamic AMPK activity. We have shown that rat and mouse hypothalamic AMPK activity is stimulated with both central (intracerebroventricular, icv) and peripheral (intraperitoneal, ip) ghrelin treatment [24, 61]. Andersson et al. have also shown the stimulatory effect of ip ghrelin injection on rat hypothalamic AMPK activity. The group examined the effect of altering hypothalamic AMPK activity on food intake using 5-amino-4-imidazole carboxamide ribonucleotide (AICAR), which can be taken up by cells and phosphorylated into ZMP, an AMP mimetic capable of activating AMPK [59]. Rats injected with AICAR showed a significant increase in food intake, thus providing the first evidence that pharmacological activation of hypothalamic AMPK stimulates food intake [59].

We have suggested that an intact cannabinoid-CB1 signalling pathway is necessary for the stimulatory effects of ghrelin on hypothalamic AMPK activity and food intake and for the inhibitory effect of ghrelin on PVN function. We provided

the first evidence that cannabinoids stimulate hypothalamic AMPK [24] and have identified cannabinoids as an important link between ghrelin, AMPK and appetite [61]. Ghrelin significantly increased hypothalamic 2-AG content of WT mice [61]. We investigated the interactions between ghrelin and the cannabinoid system in appetite regulation by in vivo studies using rimonabant, a known antagonist of the CB1 receptor, and a genetic approach using CB1 receptor knockout (CB1-KO) mice [61]. These investigations established the critical role of CB1 in mediating the effects of ghrelin on AMPK and appetite. The stimulatory effects of ghrelin on cannabinoid content, hypothalamic AMPK activity and appetite were abolished both in the absence of the CB1-KO or in the presence of rimonabant. The concordance between the effects of rimonabant and the findings in CB1-KO animals at the level of food intake, AMPK activity and cannabinoid content has highlighted the suggestion that the effect of ghrelin on both AMPK and appetite is clearly dependant on an intact cannabinoid pathway [61]. In combination, these findings suggest that the effect of ghrelin involves an increase in 2-AG synthesis and the subsequent stimulation of the cannabinoid receptor, CB1. The increase in hypothalamic AMPK activity following CB1 receptor activation ultimately leads to an increase in appetite [61].

Ghrelin has been shown to induce Ca^{2+} signalling in NPY neurons in the ARC nucleus [65]. Anderson et al. have recently shown that CaMKK2, an AMPK upstream kinase which is regulated by intracellular Ca^{2+} levels, may mediate the stimulatory effect of ghrelin on hypothalamic AMPK activity and food intake [66]. Systemic ghrelin injection increased food intake in WT animals, but failed to do so in the CaMKK2 knockout (CaMKK2-KO) mice and hypothalamic AMPK activity was significantly reduced in the CaMKK2-KO mice [66]. CaMKK2 appears to have a significant role in the orexigenic NPY/AgRP neurons, but not in the anorexigenic POMC neurons as CaMKK2-KO mice showed reduced expression of NPY and AgRP, but no differences in POMC expression. Furthermore, STO-609, a selective CaMKK2 inhibitor, inhibited NPY expression and reduced food intake and body weight in WT animals [66].

Evidence suggests that nutrient-related metabolic pathways, such as fatty acid metabolism, may act as direct modulators of the hypothalamic control of feeding [67–70]. AMPK is an important regulator of the fatty acid biosynthetic pathway [71]. Following activation, AMPK phosphorylates and inhibits ACC, preventing the production of malonyl-CoA. This further stimulates carnitine palmitoyltransferase 1 (CPT1) [72], ultimately promoting mitochondrial fatty acid oxidation and production of reactive oxygen species (ROS) [73]. The downstream effects of AMPK activation in the regulation of feeding have been proposed by Lopez et al., who recently showed that ghrelin regulates feeding through an AMPK-dependent inhibition of the de novo fatty acid synthesis pathway [72]. They have demonstrated that both fasting and ghrelin administration lead to stimulation of phosphorylation of hypothalamic AMPK, and of its downstream substrate ACC, resulting in a decrease in hypothalamic malonyl-CoA levels [72]. The authors confirmed that the orexigenic effect of ghrelin is AMPK dependent as compound C, an inhibitor of AMPK, completely prevented ghrelin's orexigenic effect, and adenoviruses

harbouring α1/α2 dominant negative AMPK were able to impair the stimulatory effect of ghrelin on food intake. Treatment with compound C resulted in activation of ACC and consequent elevation of malonyl-CoA levels [72]. Ghrelin acutely increased CPT1 activity and this corresponded to the reduction in malonyl-CoA levels [72]. This suggests that the stimulatory action of ghrelin on CPT1 activity may be mediated by simultaneous changes in malonyl-CoA levels. Inhibition of hypothalamic CPT1, by etomoxir, decreased the orexigenic effect of ghrelin, demonstrating that the stimulatory effect of ghrelin on food intake involves the activation of CPT1 [69, 72].

A recent study by Andrews et al. shows that ghrelin is able to stimulate hypothalamic mitochondrial respiration and increase UCP mRNA expression and ultimately appetite. The authors propose that ghrelin-triggered ROS production promotes uncoupling protein 2 (UCP2) mRNA expression and activity [73]. UCP2 knockout (UCP2-KO) mice did not increase food intake in response to hypothalamic ghrelin treatment. However, the effect of ghrelin on AMPK activity remained intact in UCP2-KO mice indicating that AMPK lays upstream of UCP2. Nevertheless, AMPK requires UCP2 for its effect on feeding as the effect of the AMPK activator AICAR on food intake is lost in UCP2-KO mice. Ghrelin induced NPY/AgRP mRNA expression, and activation of NPY/AgRP neurons was also UCP2 dependent [73].

Although the results from Andrews et al. [73] provide an interesting mechanism, linking ghrelin-induced changes in fatty acid metabolism and neuropeptide expression, how alterations in mitochondrial function lead to nuclear transcriptional events remains unresolved. Most recently Lage et al. have confirmed that central administration of ghrelin inactivates de novo fatty acid synthesis, stimulates hypothalamic CPT1 activity, increases NPY/AgRP mRNA expression and thus increases food intake [74]. They suggest that ghrelin stimulates NPY/AgRP mRNA expression through modulation of the transcription factor brain-specific homeobox transcription factor (BSX). BSX is a recently discovered transcription factor that increases both NPY and AgRP expression [75, 76]. The stimulatory effect of ghrelin on BSX and NPY/AgRP mRNA expression has been shown to be CPT1 dependent, as blockage of hypothalamic fatty acid β oxidation by the CPT1 inhibitor etomoxir prevented the ghrelin-induced increase in BSX, NPY and AgRP and reduced ghrelin's effect on food intake [74].

In summary, the signalling pathway of ghrelin's orexigenic effect in the hypothalamus has been further elucidated with the identification of the endocannabinoid system [24, 61] and CaMKK2 [66] as upstream to AMPK activation, while the fatty acid pathways [72] and mitochondrial UCP2 [73] have been identified as novel downstream effectors. Combining the recently described data, we put forward a pathway by which ghrelin could stimulate appetite: ghrelin–GHS-R–Ca^{2+}–endocannabinoids–CB1–Ca^{2+}–CaMKK2–AMPK–malonyl-CoA–CPT1–β-oxidation–ROS–UCP2–BSX-NPY/AgRP–food intake [71] (Fig. 5.3).

Although the above studies provide convincing evidence of short-term ghrelin effects on hypothalamic fatty acid metabolism, it appears that ghrelin plays a dual time-dependent role in modulating hypothalamic lipid metabolism. In the short-term central ghrelin elicits AMPK-mediated inactivation of fatty acid metabolism pathway,

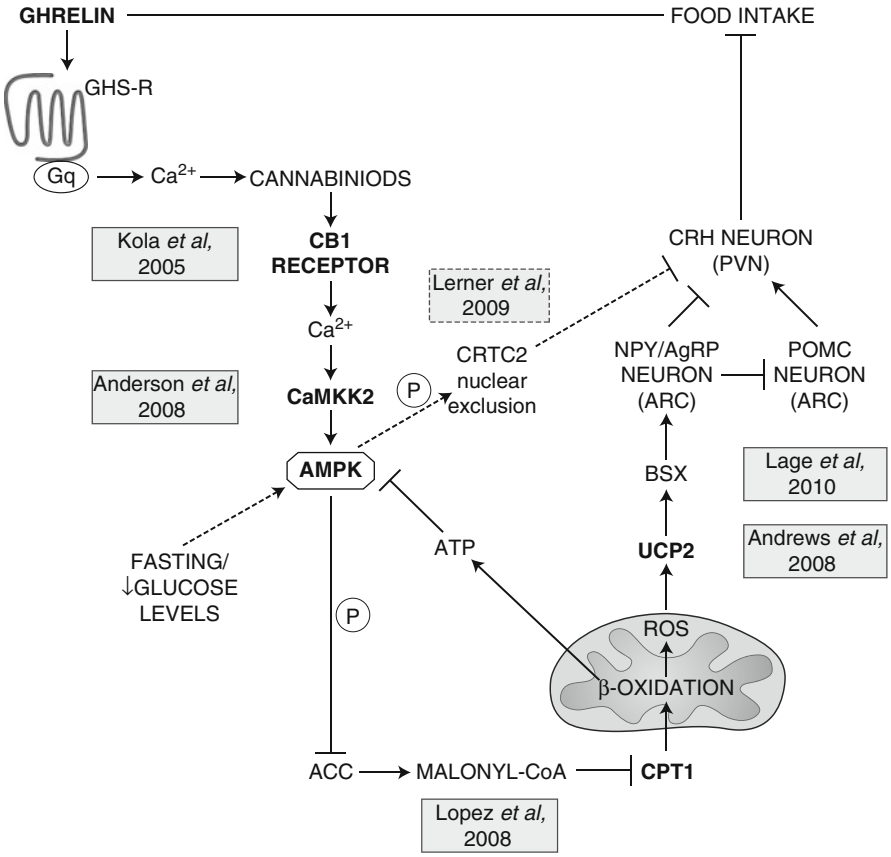

Fig. 5.3 Schematic diagram showing the proposed molecules involved in the appetite-inducing effect of ghrelin. Although the data on CRTC2 is linked to AMPK and CRH [62], it is not clear whether CRTC2 phosphorylation is affected by ghrelin or whether this molecule is involved in the CPT1–UCP2–NPY pathway. How alterations in mitochondrial function lead to BSX-mediated NPY/AgRP transcription is uncertain, but may occur via UCP2 which is critical for ghrelin-induced NPY/AgRP expression. This figure was drawn based on the studies of Andersson et al. [59], Kola et al. [24, 61], Anderson et al. [66], Lopez et al. [72], Andrews et al. [73], Lage et al. [74] and Lerner et al. [62]. *GHS-R* GH secretagogue receptor, *CaMKK2* calmodulin kinase kinase 2, *CB1* cannabinoid receptor type 1, *AMPK* AMP-activated protein kinase, *P* phosphorylation, *CRTC2* (also referred to as TORC2) CREB-regulated transcription coactivator 2, *ACC* acetylcoenzyme A carboxylase, *malonyl-CoA* malonyl coenzyme A, *CPT1* carnitine palmitoyl transferase 1, *ROS* reactive oxygen species, *UCP2* uncoupling protein 2, *BSX* brain-specific homeobox transcription factor, *NPY* neuropeptite Y, *AgRP* agouti-related peptide, *POMC* pro-opiomelanocortin, *ARC* hypothalamic arcuate nucleus, *CRH* corticotropin-releasing hormone, *PVN* hypothalamic paraventricular nucleus. → stimulation; ⊣ inhibition. Adapted from [71]

and thus promotes food intake. However, in the long term, ghrelin does not induce AMPK-dependent changes in hypothalamic fatty acid metabolism, suggesting a lack of role for the ghrelin–AMPK interaction in the long-term feeding control [77].

The nonacylated form of ghrelin, des-acyl ghrelin (or desoctanoyl ghrelin), circulates in amounts far greater than the acylated form [48]. Although various biological effects of des-acyl ghrelin have been reported [78–80], this form of ghrelin cannot activate the functional GHS-R1a receptor [79, 81] and is unable to stimulate GH release [78]. We have demonstrated that des-acyl ghrelin does not have an effect on hypothalamic AMPK activity [24] and this supports the human data showing the lack of effect of des-acyl ghrelin on appetite [82].

Ghrelin Regulation of AMPK in Cardiac Muscle

Ghrelin and GHS-R1a mRNA have been detected in human myocardium [83] and protein expression has been found in isolated murine and human cardiomyocytes [84]. Ghrelin improves cardiovascular function and is known to have direct cardioprotective effects on the ischaemic heart, where it acts to reduce damage to myocytes [78, 85–88]. It has also been suggested that early ghrelin treatment after myocardial infarction prevents an increase in cardiac sympathetic tone and reduces mortality [89]. Ghrelin administration to humans has shown beneficial effects; gain in left ventricular mass and an increase in stroke volume were observed in healthy volunteers and in patients with heart failure [90–93]. In patients with obesity, type 2 diabetes mellitus and polycystic ovarian syndrome, the low levels of ghrelin may potentially contribute to heart failure [24]. The cardio-protective effects of ghrelin appear to be direct and independent of GH release as they were also observed in both hypophysectomised rats and in in vitro studies [78, 84, 94]. The possibility has been raised that ghrelin improves cardiac function through an anti-inflammatory effect [90–93].

We found a large increase in phosphorylation and activity of AMPK in rat cardiac muscle in response to ghrelin treatment, providing the first suggestion that beneficial effects of ghrelin on the heart occur via AMPK activation [24]. It has been shown that ablation of the AMPK upstream kinase LKB1 in the heart leads to energy deprivation and impaired cardiac function [95] and cardiac-specific deletion of LKB1 leads to hypertrophy and dysfunction [96], whilst up-regulation of LKB1 and AMPK signalling was shown to improve myocardial perfusion in diabetic mice [97]. AMPK also confers protection against tumour necrosis factor α (TNFα)-induced cardiac cell death [98]. Studies using mice expressing a dominant negative AMPK mutant in the heart support the evidence that suggests that the presence of AMPK protects cardiac ATP levels [99].

In the heart, several beneficial effects of ghrelin are compatible with an AMPK-induced effect [100]. Ghrelin increases myocardial glucose uptake as evidenced by the significant rise in myocardial glycogen content [101]. The biochemical mechanism involves increased glucose transporter 4 (GLUT4) translocation [102], and increased phosphorylation of the heart-specific 6-phosphofructo-2-kinase (PFK2) which activates production of ATP by glycolysis under anaerobic conditions [103]. Activation of AMPK during ischaemia also lowers malonyl-CoA and thus increases

ATP generation via fatty acid oxidation [104]. The increased AMPK protects cardiac ATP levels and reduces infarct size and damage to myocytes during ischaemia [99]. Transitory activation of AMPK at reperfusion protects the ischaemic-reperfused rat myocardium against infarction [105]. On the other hand, prolonged AMPK activation during reperfusion can be disadvantageous, as the increased glycolysis and increased fatty acid oxidation could lead to reduced glucose oxidation and proton accumulation [104]. Reduced levels of malonyl-CoA following AMPK activation, and thus high levels of fatty acid oxidation during and following ischaemia, can contribute to ischaemic injury secondary to an inhibition of glucose oxidation, which results in a decrease in cardiac efficiency [106].

Ghrelin Regulation of AMPK in the Liver

Ghrelin binding sites [107], but not GHS-R1a, have been detected in the liver, and ghrelin has direct peripheral actions in this organ. Using a rat hepatoma cell line, H4-II-E cells, ghrelin was shown to reverse the down-regulating effect of insulin on mRNA levels of PEPCK, the rate-limiting enzyme of gluconeogenesis [108]. By opposing the effect of insulin on the expression of PEPCK, ghrelin promotes hepatic gluconeogenesis, and favours reduction in glucose uptake and fatty acid oxidation [109]. In addition, ghrelin stimulates de novo hepatic lipogenesis in a GH-independent fashion [110]. In rodent liver, ghrelin increased transcript levels of ACC and FAS thus induced a lipogenic pattern of gene expression and increased triglyceride content [111]. AMPK inhibits both gluconeogenesis and lipogenesis. Using western blotting and a functional kinase assay, we have shown that ghrelin reduces AMPK phosphorylation and inhibits AMPK activity in the liver [24]. Our data was confirmed by Barazzoni et al., who found reduced phosphorylated AMPK in the liver of ghrelin-treated rats [111]. Thus, inhibition of hepatic AMPK activity may be a mechanism through which ghrelin exerts its gluconeogenic and lipogenic effect on the liver.

Hepatic AMPK lowers systemic glucose levels by inhibiting hepatic glucose output. AMPK has been shown to suppress hepatic gluconeogenesis by blocking the expression of key gluconeogenic genes PEPCK and G6Pase [112]. Deletion of LKB1 in liver results in almost a complete loss of hepatic AMPK activity, and hyperglycaemia with elevated gluconeogenic gene expression [26]. In LKB1-deficient hepatocytes, the transcriptional co-activator CRTC2 was markedly dephosphorylated and thus transported to the nucleus, and this was associated with a dramatic increase in the expression of PGC1α and its gluconeogenic target genes PEPCK and G6Pase [28]. In terms of hepatic lipid metabolism, AMPK phosphorylates and inactivates ACC, and thus inhibits short-term fatty acid synthesis [113]. In the long term AMPK reduces expression of SREBP1c, a transcription factor responsible for regulating genes required for de novo lipogenesis [114]. Interestingly, overexpression of AMPK has been shown to ameliorate fatty liver changes in hyperlipidemic diabetic rats [115].

Ghrelin Regulation of AMPK in Adipose Tissue

Ghrelin plays an important role in adipose tissue metabolism. Ghrelin reduces the use of fat as a metabolic fuel by decreasing fat oxidation, and chronic administration of ghrelin has been shown to promote adipose tissue deposition and increase body fat content [116]. This occurs despite the GH-releasing effect; GH is well known to be a lipolytic stimulus [117]. Using a rat model of growth hormone deficiency (GHD), Sangiao-Alvarellos et al. demonstrated that chronic central ghrelin administration regulates adipose lipid metabolism, in a GH-independent manner [110]. Interestingly, ghrelin-treated animals pair-fed to saline-treated animals do not increase their weight but increase their fat tissue content as assessed by MRI, suggesting a specific effect independent of food intake and weight gain [78]. Ghrelin binding sites have been detected in human adipose tissue [107], and RT-PCR has confirmed the expression of GHS-R1a in rat adipose tissue [118]. Genetic suppression of GHS-R using transgenic expression of antisense GHS-R mRNA results in decreased adipose tissue content and activation of brown adipocyte thermogenesis in rats, indicating the importance of ghrelin and its receptor in adipose tissue energy storage [119].

A recent study using loxTB-GHS-R mice, in which transcriptional blockade prevents expression of GHS-R, has shown ghrelin to increase abdominal adiposity via GHS-R-dependent lipid retention [120]. Local acylation of ghrelin (GOAT has been detected in adipose tissue [121, 122]) could theoretically explain the GHS-R-dependent mechanism, although a GHS-R-independent mechanism could also be operating [123]. Interestingly, several studies have shown effects of both ghrelin and des-acyl ghrelin in adipose tissue. In differentiated human omental adipocytes, incubation with both acylated and des-acyl ghrelin significantly increased SREBP1 mRNA levels, as well as several fat storage-related proteins, including ACC, FAS and lipoprotein lipase (LPL) leading to stimulation of intracytoplasmatic lipid accumulation [124]. Ghrelin greatly increased lipid droplet amount in 3T3-L1 preadipocytes and induced proliferation and differentiation of 3T3-L1 preadipocytes into mature adipocytes [125].

Ghrelin has been implicated in promoting lipid storage in adipose tissue and this is consistent with its inhibitory effect on AMPK activity in this tissue [24]. In adipose tissue AMPK inhibits fatty acid and triglyceride synthesis. AMPK phosphorylates and inactivates ACC, and thus reduces levels of malonyl-CoA, a key molecule in the lipogenic pathway and an inhibitor of fatty acid β-oxidation. Therefore, activation of AMPK results in a simultaneous decrease in lipogenesis and an increase in fatty acid β-oxidation [126]. Using the AMPK activator AICAR and overexpression of constituently active AMPK, the effect of AMPK on inhibition of ACC by direct phosphorylation was shown, respectively, in cultured 3T3-L1 adipocytes and rodent adipocytes [127]. In addition, activation of AMPK in adipocytes is concomitant with a decreased expression of lipogenic enzyme mRNA [128]. A recent study found reduced AMPK activity in visceral adipose tissue samples from patients with morbid obesity; this correlates with the deleterious obesity-related changes seen in this tissue type [129].

In addition to the direct effects of ghrelin on adipose tissue, evidence suggests that ghrelin may stimulate adiposity via activation of a potential CNS-adipose tissue neuro-humoral circuit. Ghrelin is known to activate hypothalamic NPY neurons stimulating NPY gene expression, and icv infusion of this neuropeptide was shown to promote adiposity [130]. Central ghrelin treatment increased food intake and independently regulated adipocyte metabolism [131]. The authors propose that a neuroendocrine network in the CNS involving ghrelin, its hypothalamic target neurons and the sympathetic nervous system directly regulates energy metabolism in adipocytes. In white adipocytes central ghrelin favoured glucose and triglyceride uptake, increased lipogenesis and inhibited lipid oxidation, whilst in brown adipocytes central ghrelin decreased the expression of uncoupling proteins, which usually contribute to energy dissipation [131]. UCP2 promotes fat oxidation and restricts ghrelin-induced lipogenesis, and ghrelin treatment in UCP2-KO mice results in gene expression profile that favours lipogenesis [132]. The effect of central ghrelin treatment on peripheral metabolism, via the sympathetic nervous system, could be regulated by the stimulatory effect of ghrelin on hypothalamic AMPK activity. Hypothalamic cannabinoids could also be part of this neuronal circuit and mediate the effect of central ghrelin on hypothalamic AMPK activity via the CB1 receptor, as icv rimonabant was able to block the effect of central ghrelin on AMPK activity in adipose tissue (our unpublished data).

Future Directions

The role of AMPK as a homeostatic regulator of energy balance, both at the cellular and for the whole body levels, stimulates deep interest in research to develop suitable AMPK modulators to combat numerous pathologies. Due to the universal activity of AMPK, tissue specificity is essential for consideration of drug targeting. Development of tissue-specific AMPK activators and/or inhibitors for the hypothalamus, liver and adipose tissue would be of great therapeutic interest in the metabolic syndrome, obesity and type 2 diabetes, whereas cardio-specific AMPK activators could be beneficial for the ischaemic heart. If these studies can be extended to humans, key basic questions remain to be answered regarding upstream molecules and kinases and their contribution to hormonal and metabolic regulation of AMPK and further delineation of downstream targets is required. The CB1 receptor and possibly cannabinoids have been implicated as upstream components in the ghrelin regulation of AMPK, and thus provide additional therapeutic targets for negating the pathological effects of this brain–gut peptide hormone.

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Chapter 6
Ghrelin: Neuropeptide Regulator of Metabolism

Pablo J. Enriori, Zane B. Andrews, and Michael Alexander Cowley

Abstract Ghrelin regulates hypothalamic circuits to increase food intake by modulating the activity of two neuronal populations in the arcuate nucleus of hypothalamus. Those neurons (NPY/AgRP and POMC) are considered "first-order" sensory neurons in the control of food intake. Ghrelin simultaneously stimulates orexigenic NPY/AgRP neuronal activity and suppresses POMC neuronal activity via inhibitory γ-aminobutyric acid (GABA)-eric inputs from active NPY/AgRP neurons. Recent data suggests that metabolic status regulates the function of ghrelin on energy homeostasis and neuronal function and it appears that ghrelin is not able to activate this neuronal circuit in diet-induced obese mice. Contrary to expectations from 10 years ago, ghrelin seems to be more important for the regulation of blood glucose levels during starvation than it is for acute regulation of food intake. This chapter establishes the hypothesis that ghrelin primarily functions during negative energy balance to promote survival. Consistent with this idea, during calorie restriction, ghrelin increases blood glucose and suppresses glucose-stimulated insulin secretion from the pancreas. This important adaptive mechanism prevents insulin-driven clearance of glucose from the blood during negative energy balance and thus maintains an immediate energy supply.

Introduction

Ghrelin is produced by the stomach and is unique in requiring acylation for biological activity. It was originally identified as the endogenous agonist at the growth hormone secretagogue receptor (GHSR1a) and thought to regulate growth hormone

P.J. Enriori, Ph.D. • Z.B. Andrews, Ph.D. • M.A. Cowley, Ph.D. (✉)
Department of Physiology, Monash University, Building 13F, Wellington Road, Clayton, VIC 3800, Australia
e-mail: michael.cowley@monash.edu

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(GH) secretion. Subsequently, it was found to regulate hypothalamic circuits to increase food intake and was proposed as a mechanism by which the empty stomach drove appetite and food seeking. Contrary to expectations from 10 years ago, ghrelin seems to be more important for the regulation of blood glucose levels during starvation than it is for acute regulation of food intake.

Ghrelin

Ghrelin is a 28 amino acid peptide that was discovered as the endogenous ligand for the GHSR1a and is predominantly synthesized in the stomach, where it is secreted into the circulation. It is a potent stimulator of growth hormone release and enhances feeding and weight gain to regulate energy homeostasis. Pro-ghrelin requires post-translational acylation with *n*-octanoic acid or *n*-decanoic acid at the third serine for its biological activity at the GHSR1a. Thus, ghrelin exists as two forms in the plasma, acylated ghrelin and des-acylated ghrelin. Ghrelin O-acyltransferase (GOAT) is the enzyme responsible for pro-ghrelin acylation [1] and is also found predominantly in the stomach and digestive tract [1, 2]. In the stomach and duodenum GOAT co-localizes with ghrelin-expressing cells [3], where it can readily acylate newly synthesized pro-ghrelin. GOAT can acylate pro-ghrelin with other fatty acid substrates besides octanoate and this is likely regulated by fatty acid availability [4]. Once pro-ghrelin is acetylated, it is transported to the Golgi apparatus and cleaved by prohormone convertase 1/3 (PC 1/3) to form 28-amino-acid mature ghrelin [5]. Plasma des-acyl ghrelin levels are about fourfold higher than acyl ghrelin [6], but the mechanisms that control the rate at which acyl-ghrelin becomes des-acylated are unknown.

Although des-acyl ghrelin is at high concentrations in the plasma, it does not activate GHSR1a. The GHSR1a is the only functional ghrelin receptor that has been effectively characterized. It is a G protein-coupled 7-transmembrane receptor and is required to elicit growth hormone release or a food intake response to exogenous administered ghrelin.

Ghrelin Activates Hypothalamic Circuits that Control Food Intake

Neural circuits within the hypothalamus and the brainstem can both regulate food intake and it is thought that hypothalamic neurons also integrate signals of long-term energy balance such as leptin. However, brainstem circuits also integrate leptin signals [7–9]. The most studied neural circuits that regulate energy balance are in the arcuate nucleus of the hypothalamus. In the (ARC), orexigenic neurons express NPY and AgRP and anorexigenic neurons express POMC, α-MSH (one of the peptide products of POMC), and cocaine- and amphetamine-regulated transcript

(CART). These two neuronal populations in the ARC are considered "first-order" sensory neurons in the control of food intake; they receive, coordinate, and respond to changes in varying humoral factors, such as hormones, glucose, and fatty acids, associated with different metabolic states. Both NPY/AgRP and POMC neurons project to the paraventricular nucleus (PVN) and other hypothalamic nuclei. The importance of NPY/AgRP and POMC neurons in energy balance is highlighted by gene deletion experiments (see Fig. 6.1). Conditional deletion of NPY/AgRP neurons during adulthood in the ARC, using the human diphtheria toxin targeted to the AgRP locus, results in a rapid reduction in food intake and body weight [10, 11]. Deletion of POMC neurons in adulthood produced a gradual increase in food intake and body weight [10]. Embryonic deletion of either has a much more subtle effect.

Indeed, ghrelin induces feeding by robustly stimulating NPY and AgRP neuronal activity as assessed by electrophysiology [12, 13], c-fos immunoreactivity [12, 14, 15], peptide secretion [16], or gene expression [17–20]. Ablation of AgRP in adulthood abolishes the orexigenic effects of ghrelin [21] and double NPY/AgRP knockout mice do not increase food intake in response to ghrelin [17]. Consistent with the effect of ghrelin on NPY and AgRP neuronal activity, the GHSR1a is expressed on >90% of all NPY neurons in the ARC [22]. However, the GHSR1a is only expressed on <8% of POMC neurons [22]. In the ARC, the GHSR1a is also expressed on growth hormone-releasing hormone neurons [22, 23] and tyrosine hydroxylase neurons [24, 25].

At the same time that ghrelin stimulates orexigenic NPY/AgRP neuronal activity, POMC neuronal activity is suppressed via inhibitory γ-aminobutyric acid (GABA)-eric inputs from active NPY/AgRP neurons [13]. Deletion of the vesicular GABA transporter in AgRP neurons removes the inhibitory tone onto postsynaptic POMC cells, allowing unopposed activation of the melanocortin system and subsequent anorexia [26]. GABA-mediated electrophysiological inhibition of POMC neurons by NPY/AgRP neurons is accompanied by changes in POMC neuronal synaptic plasticity, in which ghrelin increases the number of inhibitory perikaryal synapses on POMC neurons [12]. Increased GABAergic inhibitory inputs on POMC neurons favor elevated food intake by lowering anorexigenic POMC neuronal activity.

How Does Ghrelin Activate NPY Neurons?

Recent evidence has begun to unravel how ghrelin activates NPY neurons to initiate changes in feeding behavior. A unique intracellular signaling modality connects mitochondria-mediated effects of G-coupled receptors to neuronal function and associated behavior. Both intraperitoneal (ip) or intracerebroventricular (icv) ghrelin injection increased AMPK phosphorylation and activity in the hypothalamus [12, 27, 28] and increased food intake. Inhibition of AMPK activity with compound C reduced ghrelin-stimulated food intake and ghrelin does not activate AMPK in GHSR −/− mice [12, 29].

In order to activate AMPK activity, ghrelin binds to the GHSR and initiates Ca^{2+} release in identified NPY neurons [30–32]. The increase in intracellular Ca^{2+} interacts

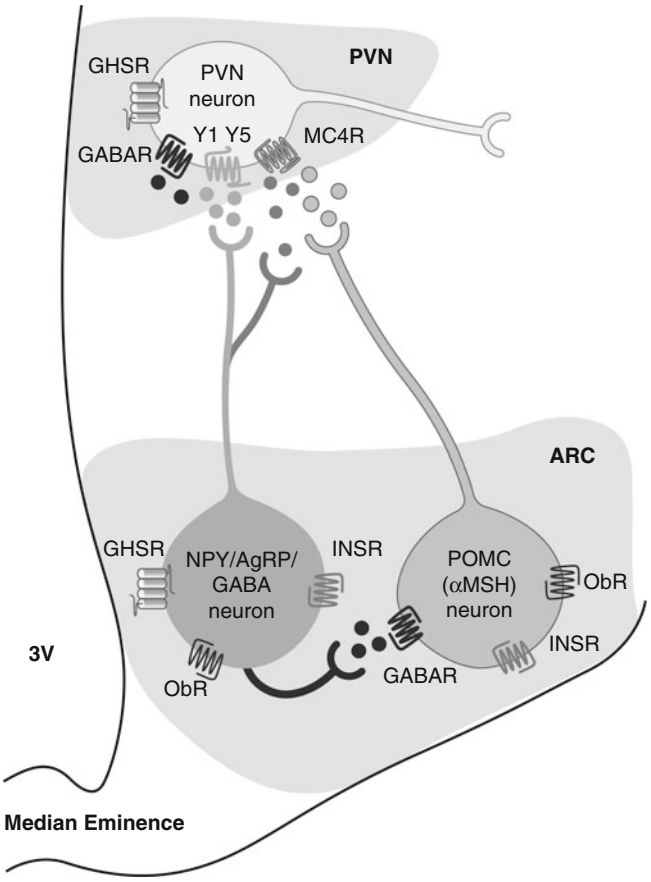

Fig. 6.1 Hypothalamic ARC–paraventricular nucleus (PVN) circuits controlling food intake and body weight regulation. NPY, AgRP, and GABA are all coexpressed in neurons of the ARC. These neurons act at downstream receptors in the PVN to stimulate food intake. POMC (*orange*) neurons that produce the anorectic alpha melanocyte-stimulating hormone (αMSH) peptide are also housed in the ARC. Increased activity of POMC neurons elevates αMSH in the PVN, which in turn acts on melanocortin 4 receptor (MC4R)-containing neurons in the PVN to suppress food intake. NPY acts on Y1 and Y5 receptors in the PVN to stimulate food intake, whereas AgRP antagonizes MC4R and prevents the anorectic actions of αMSH. Efferent outputs from the PVN project to numerous areas in the brain and brainstem to coordinate feeding behavior, energy expenditure, and adiposity. Inhibitory GABA inputs from NPY/AgRP neurons that synapse onto POMC neurons within the ARC to suppress the anorectic effects of αMSH secreted from POMC neurons. Mice engineered to prevent GABA release from NPY/AgRP neurons show a lean anorectic phenotype, illustrating that GABA maintains hypothalamic orexigenic tone. NPY neurons respond to circulating hormones and contain many receptor hormones including the ghrelin receptor (GHSR), the insulin receptor (INSR), and the leptin receptor (ObR). It is important to note that POMC neurons do not express the GHSR (<8%). Neurons in the PVN also contain GHSRs, although the neurochemical phenotype of GHSR-containing neurons in the PVN is unknown. *3V* third ventricle. This image is reproduced with permission from [36], Karger, Basel, Switzerland

with calmodulin (CaM) to activate CaM-dependent protein kinase kinases (CaMKK). Because CaMKK is an upstream kinase that can activate AMPK [33], Anderson et al. used CamKK −/− mice to show that ghrelin did not stimulate feeding [34]. Moreover, the results showed that a rise in intracellular Ca^{2+} caused CaMKK activation, which in turn led to AMPK phosphorylation and increased NPY protein and message [34]. Taken together, these studies suggest that ghrelin increases intracellular Ca^{2+}, leading to activation of CaMKK and subsequent AMPK phosphorylation.

The downstream intracellular actions after ghrelin-induced AMPK activation involve phosphorylation of acetyl CoA carboxylase (ACC), which causes the suppression of malonyl CoA and disinhibition of carnitine palmitoyl transferase 1 (CPT1). Because ghrelin activates AMPK, which is upstream of CPT1, it was hypothesized that CPT1 mediates ghrelin-induced food intake [12, 29] through a malonyl CoA-dependent mechanism. Ghrelin suppressed malonyl CoA and increased CPT1 activity and protein in the hypothalamus after 2 h, but not 6 h. Furthermore, pharmacological inhibition of CPT1 prevented ghrelin-induced food intake [12, 29]. Inhibition of CPT1 also prevented ghrelin's ability to increase NPY and AgRP mRNA expression in the hypothalamus [12].

CPT1 transports fatty acid Acyl-CoA into mitochondria for oxidation; therefore ghrelin-induced activation of the AMPK–CPT1 axis should lead to subsequent changes in mitochondrial respiration. Indeed, ghrelin stimulated palmitate-driven uncoupled respiration in isolated hypothalamic mitochondria [12] in a UCP2-dependent fashion, as no effect was observed in UCP2−/− mice. Moreover, AMPK is required to activate this UCP2-dependent mitochondrial mechanism and UCP2 is required to permit CPT1 activation. Upon binding to its receptor, ghrelin activates this AMPK–CPT1–UCP2 axis and initiates a mitochondrial mechanism that is essential for mitochondrial biogenesis in NPY/AgRP neurons, electrical activation of NPY/AgRP neurons, and ghrelin-triggered synaptic plasticity of POMC. Collectively, this causes ghrelin-induced food intake.

Reactive oxygen species (ROS) are a by-product of enhanced mitochondrial respiration during fatty acid oxidation and UCP2 is a mitochondrial protein primarily known to buffer or scavenge excessive ROS production [35]. It was discovered that UCP2, specifically in NPY/AgRP neurons, is required to buffer excessive ROS production generated by ghrelin-induced fatty acid oxidation [12]. Thus, ghrelin activation of this AMPK–CPT1–UCP2 pathway permits increased fatty acid oxidation while buffering increased ROS in NPY neurons, but not POMC neurons because they lack GHSRs.

Diet-Induced Obesity Causes Ghrelin Resistance in NPY/AgRP Neurons

Recent data suggests that metabolic status regulates the function of ghrelin on energy homeostasis and neuronal function [16, 36]. In diet-induced obesity (DIO) mice (1) peripheral ghrelin does not stimulate food intake [37]; (2) ghrelin transport across

the blood–brain barrier is impaired [38]; (3) NPY/AgRP feeding circuits are permanently disrupted [39]; and (4) basal hypothalamic AMPK activity is suppressed [40]. Because of these events, we hypothesized that ghrelin would not stimulate NPY/AgRP neurons in diet-induced obesity.

Central ghrelin did not activate ARC neurons, as demonstrated by Fos-immunoreactivity, increase expression of hypothalamic NPY and AgRP mRNA expression, or induce feeding in either the light or the dark phases in DIO mice [16]. Further, ghrelin did not induce AgRP or NPY peptide secretion in hypothalamic explants from DIO mice compared to chow-fed controls [16]. These results clearly point to defective NPY/AgRP neuronal function in diet-induced obese mice (see Fig. 6.2). To determine whether downstream NPY/AgRP neural targets are intact, we delivered NPY directly into the lateral ventricle and this was able to induce food intake in both chow-fed and DIO mice. Thus, the inability of ghrelin to activate NPY/AgRP neurons in the ARC or stimulate peptide release at synaptic targets in the PVN is the cause of ghrelin resistance in diet-induced obesity, and targets of ghrelin-sensitive NPY neurons remain responsive to NPY and AgRP [41]. This is analogous to the situation with leptin sensing in lean and DIO mice, where leptin sensing occurs normally in the ARC of lean animals, but is lost in the arcuate nucleus neurons in DIO animals [41] and is retained in second-order neurons like the ventromedial hypothalamus [42].

Moreover, ghrelin and GOAT mRNA in the stomach, expression of hypothalamic GHSR mRNA, and acylated plasma ghrelin are all decreased in DIO mice [16]. The GHSR has high constitutive activity [43] and contributes to the basal regulation of food intake and body weight even in the absence of ghrelin ligand binding [44]. Thus, decreased hypothalamic GHSR mRNA expression may further contribute to hypothalamic ghrelin resistance due to lower basal constitutive activity [43] and less GHSR, to which ghrelin can bind.

Interestingly, central ghrelin increases plasma growth hormone in chow-fed mice, but not in DIO mice [16]. This supports our hypothesis that DIO promotes hypothalamic ghrelin resistance and further, that hypothalamic ghrelin resistance is not only confined to appetite-regulating pathways but also affects other neurons expressing GHSR.

The exact hypothalamic mechanisms behind this phenomenon are unclear, but may be related to endocrine changes that occur in DIO, such as hyperglycemia and hyperinsulinemia. Central infusion of insulin during fasting prevents upregulation of NPY mRNA expression and reduces immunoreactive NPY concentrations in the PVN [45]. Central insulin infusion also reduces both hyperphagia and overexpression of hypothalamic NPY mRNA in diabetic rats [46].

Fig. 6.2 (continued) neurons in the PVN in DIO mice despite no observable effect in the ARC. Because central ghrelin does not increase food intake in DIO, the Fos activation in the PVN can be involved in stimulating food intake. The direct actions of ghrelin in the PVN may control body weight and adiposity independently of feeding behavior. In addition to ghrelin's effects on the appetite-stimulating neurons in the ARC, ghrelin does not stimulate growth hormone-releasing hormone (GHRH) neurons and growth hormone (GH) release from the pituitary. This image is reproduced with permission from [36], Karger, Basel, Switzerland

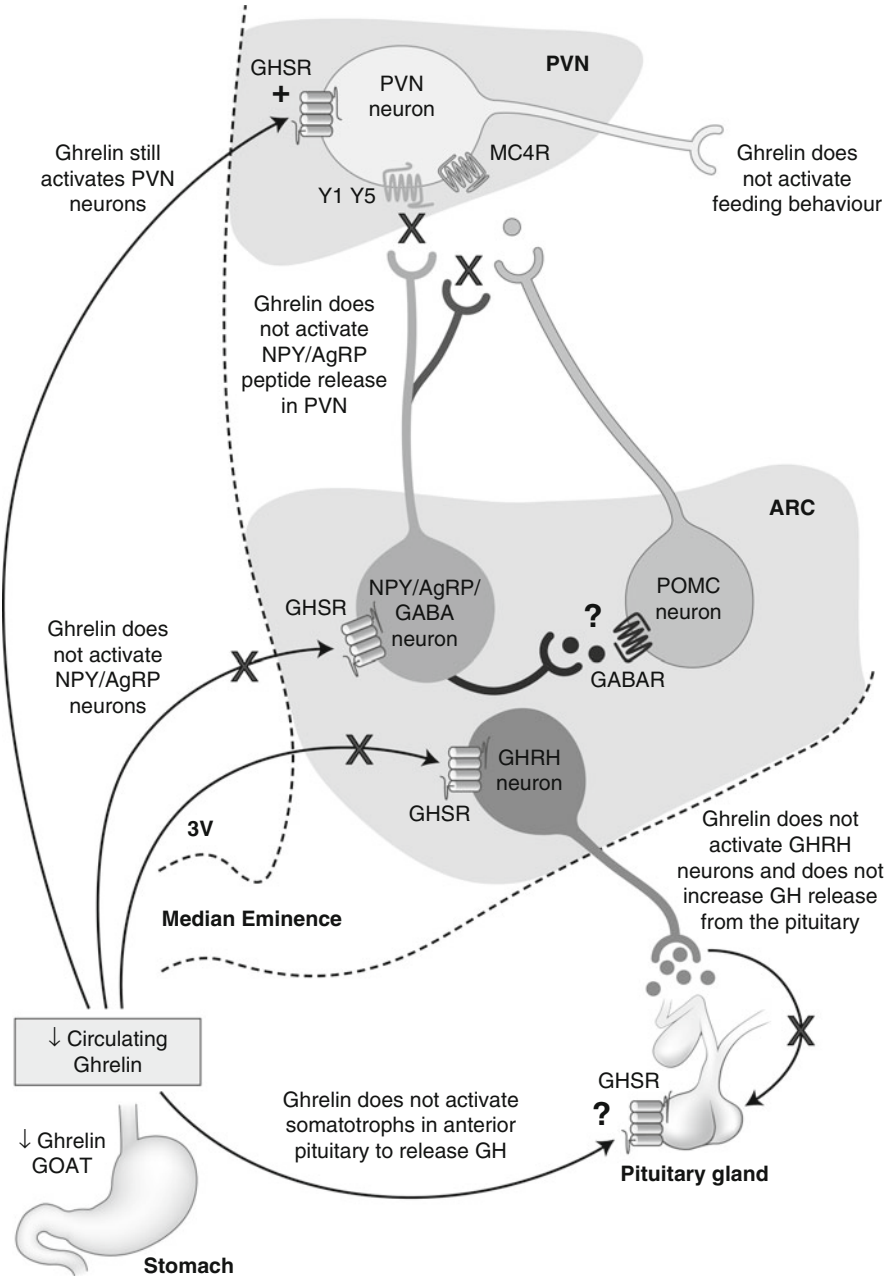

Fig. 6.2 Diet-induced obesity (DIO) causes ghrelin resistance in ARC neurons but not PVN neurons. Circulating ghrelin, ghrelin mRNA, and GOAT mRNA in the stomach and GHSR mRNA in the hypothalamus are all reduced in diet-induced obesity. Further, ghrelin does not activate NPY/AgRP neurons or NPY/AgRP peptide release and central ghrelin does not increase food intake in DIO mice. It is currently unknown whether DIO affects the ability of ghrelin to modulate POMC cell function through NPY inhibitory inputs. Interestingly, ghrelin still activates Fos-positive

Ghrelin Regulates Body Weight Independent of Food Intake

An intriguing finding from Briggs et al. was that central ghrelin injection in DIO mice still activated Fos-positive neurons in the PVN even though ARC neurons were not activated [16]. This highlights that ghrelin directly activates PVN neurons under diet-induced obese conditions and that the PVN is presumably both a "first-order" and "second-order" nucleus responding directly to ghrelin that has gained access in the cerebrospinal fluid (first order) and responding indirectly to ghrelin-induced NPY and AgRP release (second order) in chow-fed animals. In DIO conditions, we hypothesize that ghrelin increases adiposity through PVN signaling despite ghrelin resistance in the ARC. In support of this, GHSR is expressed in the PVN [25] and GHSR knockdown in the PVN in vivo (60% knockdown) did not affect daily food intake but significantly reduced body weight and blood ghrelin levels [47]. Moreover, Theander-Carrillo and colleagues showed with pair feeding experiments that chronic central ghrelin increases fat deposition independent of changes in food intake. Central ghrelin increased respiratory quotient (RQ) in both *ad libitum* and pair-fed animals, indicating greater carbohydrate metabolism. White adipose tissue mRNA levels of the fat storage-promoting enzymes lipoprotein lipase (LPL), acetyl-CoA carboxylase α (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD1) were also increased [48]. Ghrelin neutralization with the acyl-ghrelin binding compound, NOX-B11(2), during fasting–refeeding cycle impairs body weight recovery, but does not affect food consumption during refeeding in rats [49]. NOX-B11(2) treatment reduced glycolytic and lipogenic hepatic enzyme expression during fasting and also slowed enzyme expression and glycogen recovery during refeeding [49]. This study indicates that ghrelin affects hepatic energy metabolism during the fasting–refeeding cycle, independent of effects on food intake.

The effect of ghrelin on adiposity may be independent of ghrelin-induced feeding mediated by NPY/AgRP peptide release, as ghrelin still increases body weight in NPY-deficient mice [50]. Although, it should be noted that NPY ablation in *ob/ob* mice increased energy expenditure and decreased obesity [51], overexpression of NPY in the PVN resulted in obesity [52], and NPY deficiency reduces body weight gain on a high-fat diet (HFD) [53]. Thus, the hypothalamic actions of ghrelin in energy metabolism are most likely twofold: (1) ghrelin acts via NPY/AgRP neurons in the ARC to increase food intake and body weight and (2) ghrelin acts directly on GHSR-containing neurons in the PVN to increase body weight and adiposity. Further studies are required to elucidate the different functions of GHSR signaling in different hypothalamic nuclei.

Ghrelin Modulates Lipogenesis

The study by Theander-Carrillo and colleagues highlights that ghrelin regulates white adipose tissue via central hypothalamic actions. The central actions of ghrelin on adipogenesis are growth-hormone independent, as ghrelin increased fat mass,

mRNA expression, protein expression, and enzymatic activity levels in adipose tissue from growth hormone-deficient rats [48]. In addition to a central action, ghrelin also has direct actions on lipogenesis in adipose tissue. Although an early study showed that ghrelin inhibited adipogenesis in 3T3-L1 cells [54], recent cell culture studies show that both des-acyl and acyl ghrelin directly stimulate cell proliferation and adipocyte differentiation in 3T3-L1 preadipocytes by increasing adipogenic factors such as PPAR gamma and C/EBPα [55–58]. Ghrelin also inhibits adipocyte apoptosis in 3T3-L1 cells through activation of MAPK pathways. Acyl-ghrelin and des-acyl ghrelin stimulate lipid accumulation in human visceral adipocytes [59] and ghrelin directly increases leptin production in cultured rat adipocytes [60]. In vivo studies indicate that ghrelin both directly and indirectly regulates adipogenesis. The direct affects are mediated by ghrelin binding to the GHSR, which is expressed at low levels in adipose tissue [59, 61, 62]. Ghrelin increases triglyceride content in adipose tissues [63] and Davies et al. demonstrated that acyl-ghrelin increased abdominal adipose tissue in a depot-specific manner. This depot-specificity was not due to differences in GHSR expression, as expression levels were similar in ghrelin-responsive and -nonresponsive depots [61]. A similar GHSR-independent effect was observed in vivo by Thompson et al., as acyl ghrelin, but not a GHSR agonist, increased adipogenesis. In these studies ghrelin was delivered i.v., i.p., or via minipumps [64]; thus it is likely that ghrelin entered the brain and altered the central regulation of adipogenesis [48]. Although both acyl- and des-acyl ghrelin stimulate adipogenesis in cell culture models [56–59], in vivo studies show differential effects of acyl- and des-acyl ghrelin on adipogenesis. Des-acyl ghrelin is shown to increase adiposity [64], to have no effect on adiposity [61], or to decrease adiposity and improve insulin sensitivity [65]. More recent studies support the idea that des-acyl ghrelin maintains insulin sensitivity and prevents adipogenesis [66]. There is no doubt that ghrelin stimulates weight gain by increasing food intake and adiposity. However, there are many equivocal issues that need to be addressed in the future. For example, does ghrelin primarily influence adiposity through central or peripheral mechanisms? How do acyl ghrelin, des-acyl, and the ratio of acyl/des acyl ghrelin affect adipogenesis and insulin sensitivity? Understanding these critical issues will help design specific therapies to combat obesity and diabetes.

Lessons from Knockout Models

Studies from knockout (KO) models suggest that ghrelin signaling has only modest effects on body weight and food intake. Studies show that GHSR−/− mice have significantly reduced body weight on a regular chow diet [67, 68] and this difference may be related to the constitutive signaling properties of the GHSR in the absence of ligand binding [44]. However, Zigman et al. and Pfluger et al. did not witness any difference in body weight on a standard chow diet, using different GHSR−/− mouse lines [69, 74]. In order to determine the neuronal phenotype controlling body weight and food intake, Shuto et al. created transgenic rats expressing a GHSR antisense oligonucleotide under the tyrosine hydroxylase (TH) promoter.

Knocking down the GHSR on TH (dopamine) cells reduced food intake, body weight, and fat mass [24]. Due to the fact that ghrelin activates the mesolimbic dopamine pathway [70, 71], it is possible that the GHSR regulates food intake, body weight, and adiposity by enhancing the reward value of food, particularly high-fat food [72].

Recent studies in GOAT–/– mice, which produce no acylated ghrelin [73], also showed no difference in body weight gain on a chow diet and no difference in fat mass or lean. This phenotype is supported in ghrelin–/– mice, which also show no difference in body weight on a standard chow diet [67]. Further, Pfluger et al. only found a significant difference in body weight when ghrelin–/– or GHSR–/– mice were bred together to generate double-knockout mice [74].

When placed on an HFD (a well-known obesity and diabetes model) modest changes were observed: ghrelin–/– mice gained less weight and had greater energy expenditure on an HFD than their wild-type controls despite having the same food intake [75]. However, recent studies using congenic ghrelin–/– mice outbred on a C57BL/6J ($n = 10$) background showed no protection against HFD [67]. This is supported by studies in GOAT–/– mice as they gained weight on HFD at the same rate as wild-type controls [73]. GHSR–/– mice gained less weight on an HFD relative to wild-type controls. However, the cause of this lower weight gain remains unknown, as both a decrease in food intake and no change in food intake have been recorded [67, 69]. Clearly the equivocal nature of these knockout studies suggests that ghrelin signaling does not play a major role in body weight regulation in mice. Although these knockout models suggest that ghrelin plays a minimal role in food intake and body weight, it is too early to dismiss a role for ghrelin in food intake and body weight based on these knockout models. As there is a wealth of literature showing that exogenous ghrelin administration increases food intake and adiposity, an important development to understand the true physiological role of ghrelin will be the generation of a temporal ghrelin knockout mouse. This model will side step the issue of compensatory developmental mechanisms that may develop after ghrelin deletion. This is important as neonatal ablation of NPY/AgRP neurons has minimal effects on feeding, whereas ablation in adults causes rapid starvation [10, 11].

Role of Ghrelin in Glucose Homeostasis

Knockout models illustrate that ghrelin plays a major role in glucose homeostasis. The first clear evidence of an interaction between ghrelin and glucose was presented by Tschop et al., who demonstrated that a single ghrelin injection (subcutaneously) increases the RQ, suggesting that an augmented utilization of carbohydrates and reduced utilization of fat promote the weight gain observed in mice and rats under ghrelin treatment [76].

The dissociation between the role of ghrelin on the regulation of body weight (food intake and body adiposity) and glucose homeostasis was demonstrated by the generation of mutant mouse models. Indeed, the deletion of the ghrelin gene prevents the

onset of glucose intolerance generated by an HFD, despite no observed differences in either body weight or food intake between ghrelin KO mice and wild-type littermates [77]. Yet more significant were the results shown after crossing leptin-deficient (*ob/ob*) mice with ghrelin KO mice to study the interaction of ghrelin and leptin signaling. The targeted gene deletion improved glucose tolerance and augmented insulin secretion in *ob/ob* ghrelin KO mice compared with *ob/ob* mice [78]. Interestingly, there was no change in the obese phenotype. Both glucose and insulin tolerance tests revealed lower glucose concentrations in double-deficient mice compared with *ob/ob* mice. In addition, plasma glucose levels also normalized during fasting conditions.

The relevance of ghrelin in glucose homeostasis was disputed by another double-KO mice model from Pfluger et al. The authors recently showed that simultaneous deletion of ghrelin and its receptors failed to produce changes in glucose disposal when the mice were fed standard chow diet [74]. It is possible that the effect of ghrelin on glucose homeostasis is clearer when mice are on an obesogenic HFD.

Whether ghrelin directly suppresses insulin secretion to maintain physiological glucose levels remains unclear. Early studies showed that acute ghrelin treatment induced hyperglycemia and reduced insulin secretion in healthy humans [79], but not in obese patients [80]. As ghrelin strongly stimulates GH secretion [81], and GH increases blood glucose concentrations, free fatty acid uptake, and inhibition of glucose transport in skeletal muscle, the effect of ghrelin on glucose metabolism was thought to reflect increased GH secretion. To unravel the effect of ghrelin, independent of GH actions, several strategies were designed. Pretreatment with pegvisomant, a GH receptor antagonist used in acromegaly, followed by ghrelin mimetic injection induced increases in glucose and insulin levels. Moreover ghrelin infusion in humans with GH deficiency increased glucose and insulin levels, supporting the concept that ghrelin increased blood glucose via a GH-independent pathway [82, 83].

Possible Mechanism of Action

Although ghrelin is mainly produced in the stomach, it is also produced at low levels by the hypothalamus and by other peripheral tissues. Based on the current information, paracrine, endocrine, and neural mechanisms are all reasonable possibilities to explain ghrelin effects on glucose. Maintaining glucose homeostasis requires glucose sensing by the central nervous system and by peripheral tissues. We discuss some of them in the next section. For more information see reviews of [84] and [85].

Role of Ghrelin in Central Glucose Sensing

Glucosensing neurons, located in hypothalamus and brainstem, regulate the activity of the autonomic nervous system, hormone secretion, glucose production, and glucose uptake and utilization [86]. Because POMC neurons are excited by glucose

[87] and NPY neurons seem to be inhibited by glucose [88, 89], it is possible that ghrelin produced in the stomach acts on melanocortin circuits to regulate peripheral glucose homeostasis. It was also suggested that ghrelin is produced by a group of neurons adjacent to third ventricle between the dorsal, ventral, paraventricular, and arcuate nucleus of hypothalamus [13]. These ghrelin-expressing neurons project to key hypothalamic circuits that include those producing NPY, AgRP, POMC, and CRH, raising the possibility that hypothalamic ghrelin participates in systemic glucose regulation. Ghrelin may also have central NPY-mediated effects on peripheral blood glucose, as activation of NPY in the hypothalamus increased hepatic glucose production [90].

Peripheral Targets of Ghrelin to Regulate Glucose Homeostasis

Pancreas, liver, skeletal muscle, and white adipose tissue are critical to the control of lipid and glucose homeostasis. We summarize here some of the relevant information about the ghrelin effects on these tissues to act co-coordinately to regulate glucose homeostasis.

Pancreas

Early studies demonstrated that ghrelin, as well as its receptor, is expressed in the pancreatic islets (α-, β-, and ε-cells of rats and humans) [91, 92]. In vivo studies showed that ghrelin inhibits glucose-stimulated insulin secretion in a dose-dependent manner in rat pancreatic islets [92]. However, there are conflicting results about the role of ghrelin in insulin secretion. It was demonstrated that low doses of ghrelin (10^{-12} to 10^{-10}) inhibited insulin release from mouse islets, but high doses ($>10^{-8}$) stimulated insulin secretion [93]. It has been demonstrated that UCP2 modulates ATP production in pancreatic β-cells to regulate the activation of ATP-sensitive K^+ channels, Ca^{2+} influx, and insulin release in response to a glucose challenge and blockade of UCP2 augments glucose-stimulated insulin secretion in islet from DIO mice [94, 95]. Although several potential mechanisms are suggested for ghrelin action on pancreatic β-cells, it was demonstrated that ablation of ghrelin reduces expression of UCP2 mRNA in the pancreas, which contributes to enhanced glucose-induced insulin secretion. Hence, ghrelin may regulate glucose homeostasis by chronically regulating pancreatic UCP2 expression and therefore glucose-stimulated insulin secretion [78].

A paracrine/autocrine role of the gene-derived peptides (endogenous pancreatic ghrelin) in the regulation of insulin secretion has been postulated [92], but it remains still a controversial topic [96]. The only certain evidence is that systemic infusion of ghrelin elevates blood glucose levels. While studies in animals showed an important effect of ghrelin on insulin secretion, experiments in humans are more controversial,

probably because they used fasting insulin as the marker of ghrelin effects on β-cells. Recently, Tong et al. examined the effect of continuous infusion of ghrelin on dynamic insulin secretion and glucose metabolism. Using a continuous infusion of ghrelin and intravenous glucose tolerance test, they were able to demonstrate that exogenous ghrelin markedly reduced the first-phase insulin (acute insulin response to glucose) response to intravenous glucose in healthy humans [97]. In summary, the in vitro reports are the most conflicting regarding the role of ghrelin on insulin secretion. The in vivo studies, mostly in rodents, strongly suggest an inhibitory effect of ghrelin on insulin secretion.

Liver

Earlier studies demonstrated that phosphorylation of AKT plays a key role in suppression of hepatic gluconeogenesis. Cell culture studies and in vivo experiments in rats show that ghrelin reduces insulin action on liver due to a decrease of AKT phosphorylation [98, 99] resulting in potential gluconeogenic effects. These results have not been confirmed in human subjects, since infusion of ghrelin induced peripheral resistance, but did not affect hepatic glucose production [83]. However, it is important to consider that nutritional status may influence ghrelin regulation of glucose metabolism. Recently it was demonstrated that ghrelin neutralization (using a specific Spiegelmer compound) during the fasting–refeeding cycle alters hepatic glucose and lipid metabolism in rats [49].

Skeletal Muscle

Some researchers reported that ghrelin administration in rats increased insulin sensitivity in skeletal muscle by enhancing AKT-dependent insulin signaling selectively in oxidative muscle [99]. However, studies in human subjects show the opposite results. It is important to consider that high doses of ghrelin may cause secretion of many pituitary hormones, such as GH, prolactin, and adrenocorticotrophic hormone which may influence systemic glucose homeostasis [100]. To circumvent these interferences, Vestergaard et al. studied two populations: one with healthy human subjects and the other with hypopituitarism in the absence of growth hormone and cortisol replacement therapy. It was demonstrated that a ghrelin infusion during hyperinsulinemic-euglycemic clamp acutely decreases peripheral insulin sensitivity together with stimulation of lipolysis, showing a direct ghrelin effect on muscle independent of GH action [83] in both groups.

In summary, most data supports ghrelin acting to impair insulin sensitivity, especially in the muscle. However, the underlying mechanism remains to be elucidated.

White Adipose Tissue

There are conflicting results regarding the impact of ghrelin on insulin sensitivity of adipocytes. Some studies showed a direct role of ghrelin in enhancing insulin-stimulated glucose uptake in isolated epididymal white adipose tissue [101] and adipogenesis [64]. However, other studies have shown the opposite, such as increased lipolysis [63, 83]. These contradictory results may arise from the use of different modes of ghrelin exposure and analysis of adipocytes from different locations, consistent with reports that show fat-depot-specific sensitivity to ghrelin [61]. Additional in vitro and in vivo studies are required to investigate the physiological relevance of the effects of ghrelin on glucose uptake in adipose tissue.

These studies highlight that ghrelin increases glucose production in the liver and attenuates glucose-stimulated insulin release from the pancreas. Under non-physiological conditions this may promote a diabetic phenotype [78]. However, we must consider this action of ghrelin in its physiological context. Metabolic status regulates ghrelin's action in the brain and circulating ghrelin levels [36]. Further, ghrelin levels are increased during calorie restriction in mice, rats, and humans and decreased in obesity [16, 73, 102–106].

Under 50–60% calorie restriction, mice lacking ghrelin, GHSR, and GOAT have lower blood glucose levels than their wild-type littermates [67, 73]. Recent studies in GOAT−/− mice show that an essential function of ghrelin is to maintain survival during severe calorie restriction [73]. GOAT-deficient mice lack acylated ghrelin and could not control blood glucose during severe calorie restriction. After 7–8 days of calorie restriction mice appeared moribund and had to be euthanized. Infusion of ghrelin or GH normalized blood glucose to wild-type levels. Interestingly, GOAT−/− mice showed no defect in food intake under normal dietary or calorie-restricted conditions. These studies clearly illustrate that ghrelin is required to maintain survival under conditions of severe negative energy balance, not by increasing food intake, but rather by maintaining blood glucose. In support of this notion, fasted blood glucose levels are not different in GHSR−/− mice on an HFD, but are lower in calorie-restricted GHSR−/− mice compared to wild-type controls [67].

Conclusions

Recent studies have expanded our knowledge of how ghrelin acts in both the brain and periphery to control energy metabolism. This chapter establishes the hypothesis that ghrelin primarily functions during negative energy balance to promote survival. Consistent with this idea is the proposition that ghrelin increases blood glucose and suppresses glucose-stimulated insulin secretion from the pancreas during calorie restriction. This important adaptive mechanism prevents insulin-driven clearance of glucose from the blood during negative energy balance and maintains an immediate energy supply. Whether or not ghrelin plays a role in the pathogenesis of diabetes,

by promoting hyperglycemia, remains to be determined. However, we would argue against a major role of ghrelin during diet-induced obesity. First, ghrelin peptide in the circulation as well as ghrelin and GOAT mRNA in the stomach and GHSR in the hypothalamus are all reduced in diet-induced obesity. Second, diet-induced obesity causes central ghrelin resistance at the level of the hypothalamic ARC nucleus. Therefore, we regard ghrelin as an important component in the physiological response to maintain blood glucose levels during starvation, rather than a stimulator of appetite in obese mammals.

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Chapter 7
GOAT and the Regulation of Energy and Glucose Homeostasis

Henriette Kirchner, Matthias Tschöp, and Jenny Tong

Abstract Ghrelin is the only known circulating hormone that potently stimulates food intake and adipogenesis in rodents and humans. Through a unique posttranslational modification, the premature ghrelin peptide is acyl-modified with a medium-chain fatty acid at the serine-3 position by ghrelin-*O*-acyl transferase (GOAT) to form acyl ghrelin. This step is necessary to activate ghrelin's only known receptor, the growth hormone secretagogue receptor 1a, also known as the ghrelin receptor. GOAT activity is highly influenced by the availability of dietary lipids; dietary lipids serve as a direct GOAT substrate for ghrelin acylation. Further, GOAT gene expression and circulating acyl ghrelin levels are decreased after long-term starvation when dietary lipids are absent. These findings suggest that the ghrelin-GOAT system plays an important role in linking nutrient availability with endogenous regulation of energy homeostasis, especially adipogenesis. Recent data further suggest that GOAT is a significant factor in the regulation of glucose homeostasis as specific pharmacological GOAT inhibition improves glucose tolerance and insulin secretion. During caloric restriction, the ghrelin-GOAT system may play an important role for the maintenance of physiological range glucose levels by stimulating the secretion of growth hormone.

H. Kirchner, M.S., Ph.D.
Department of Internal Medicine and Endocrinology, Metabolic Diseases Institute, University of Cincinnati, Cincinnati, OH, USA

M. Tschöp, M.D. (✉)
Department of Internal Medicine, University of Cincinnati,
2180 E. Galbraith Road, Room A-123, Cincinnati, OH 45237, USA
e-mail: matthias.tschoep@ucmail.uc.edu

J. Tong, M.D., M.P.H.
Department of Medicine, University of Cincinnati, Cincinnati, OH 45237, USA

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Introduction

Ghrelin is the only known orexigenic hormone that is released into the circulation [1]. It is produced in the periphery, mainly by the stomach [2], but the biological actions of ghrelin are predominantly mediated via pathways in the central nervous system [3, 4]. In the brain, ghrelin acts on areas that are known to regulate food intake and energy homeostasis. Specifically, ghrelin increases food intake by activating hypothalamic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons [3, 4], which are currently considered as crucial players in regulating feeding and energy metabolism [5, 6]. Additional to its orexigenic effect, ghrelin increases adipogenesis and decreases energy expenditure [1]. Ghrelin has a very characteristic secretion pattern. Under physiologic conditions, blood concentrations of ghrelin rise before a meal and are suppressed by incoming nutrients [7, 8]. However, the recovery of ghrelin levels in plasma after a meal does not seem to be an important determinant of meal intervals [9]. Due to this distinct secretion pattern and its orexigenic and adipogenic functions ghrelin has been thought of as a meal initiation factor or a hunger hormone [10].

Ghrelin has only one known receptor, the growth hormone secretagogue receptor-1a (GHSR-1a), which is mainly expressed in the pituitary and the hypothalamus of the brain [11]. In order to bind and activate its receptor ghrelin needs to be specifically acylated at its third amino acid (Serine-3) with a fatty-acid residue. Ghrelin-receptor activation is most robust, when ghrelin is acylated with one of the medium-chain fatty acids—hexanoyl (C6:0), octanoyl (C8:0), or decanoyl (C10:0) [12]. However, the most abundant form of acyl ghrelin is *n*-octanoly ghrelin [2]. Acyl ghrelin accounts for about 20% of total ghrelin in circulation. The vast majority (80–90%) of circulating ghrelin is the non-acylated des-acyl ghrelin. Since there is no known cognate receptor for des-acyl ghrelin [2], the physiological relevance for this ghrelin isoform has been questioned. However, some studies have shown that des-acyl ghrelin has orexin-mediated effects on food intake [13] and GHSR-1a-independent effects on energy and glucose homeostasis [13–15].

The enzyme that couples the fatty acid-residue to ghrelin in order to form the bioactive acyl ghrelin was discovered in the year of 2008 and was named ghrelin *O*-acyltransferase (GOAT) [16, 17]. GOAT was previously recognized as the orphan enzyme called membrane-bound *O*-acyltransferase 4 (MBOAT4), which is a member of the family of membrane-bound *O*-acyltransferases (MBOATs). MBOAT4 had no known physiological function until 2008 when two independent research groups discovered that MBOAT4 is able to acylate ghrelin [16, 17]. Consequently, MBOAT4 was renamed GOAT. However, the name of the GOAT gene remains to be *MBOAT4*. GOAT-deficient mice do not produce acyl ghrelin [16]. Therefore, it can be assumed that GOAT is the only existing enzyme that acylates ghrelin. GOAT has eight predicted membrane-bound regions and is possibly located in the membrane of the endoplasmic reticulum [17]. Consequently, GOAT is not expected to be released into the circulation.

Techniques That Led to the Discovery of GOAT

An extensive search for the ghrelin-acylating enzyme had been ongoing since ghrelin was discovered in 1999. One approach to identify this enzyme was to study the known proteins that, similarly to ghrelin, carry a fatty acid residue at the Serine-3 position. Intriguingly, there are only two known proteins in which serine-3 is acylated naturally: ghrelin and Wnt3a. The acyltransferase that acylates Wnt3a is called Porcupine and has structural similarities with members of the MBOAT family [18]. For this reason, it was speculated that the ghrelin-acylating enzyme might have structural similarities to the MBOATs, or it might even be a member of this enzyme family. As a strategy to identify the ghrelin-acylating enzyme candidate genes, known acyltransferases were selected based on the following criteria: (1) similarity to known acyltransferase sequences; (2) presence of a human homologue; and (3) function of the gene had to be unknown [16]. According to these criteria 12 [16] to 16 [17] candidate genes were selected. Most of them were orphan MBOATs. Yang et al. [17] transfected cell lines that were known not to produce any ghrelin with the ghrelin gene *ghrl*. As a result these cell lines started to produce des-acyl ghrelin. In a second step the cells were co-transfected with cDNA of known acyltransferases and other potential ghrelin-acylating candidate-enzymes. Only when cells were co-transfected with cDNA of MBOAT4 could acyl ghrelin be detected. This experiment clearly demonstrated that MBOAT4 was necessary for the acylation of ghrelin.

Gutierrez et al. [16] also used cell culture models to identify the putative enzyme that activates ghrelin. But instead of adding genes that would potentially express the acylating enzyme, they chose to use gene silencing technology to identify it. TT cells that are derived from human medullary carcinoma cells have been known to be able to produce octanoyl-ghrelin when octanoic acid is added to the cell culture medium. Therefore, it was assumed that the genetic information of the ghrelin-activating enzyme was present and could be successfully expressed in TT cells. In the next step, small interference RNA (siRNA) designed to specifically knock down genes of putative candidate-enzymes was used to inhibit production of acyl ghrelin in the TT cell-model. When silencing one candidate enzyme at a time, the group discovered that TT cells treated with siRNA of MBOAT4, but not with siRNAs of any other candidates, significantly decreased ghrelin acylation. To verify that MBOAT4 is truly the only acyltransferase that acylates ghrelin in vivo, Gutierrez et al. generated knockout (KO) mice for GOAT. These mice produced large amounts of des-acyl ghrelin but were not able to generate acyl ghrelin. The total absence of acyl ghrelin in the GOAT knockout (GOAT-KO) mice provides proof that GOAT is the only enzyme that acylates ghrelin [16].

GOAT Physiology and Mechanism of Ghrelin Acylation

GOAT is conserved across vertebrates and functional GOAT activity has been shown in humans, rats, mice, and zebra fish [16, 17]. Amino acid sequences that are similar to GOAT exist in other vertebrates, which is consistent with the knowledge

that octanoylated ghrelin is present across vertebrates [16]. GOAT has eight predicted membrane-bound regions and is possibly located in the membrane of the endoplasmic reticulum [17]. The locations of the GOAT gene expression in humans and rodents parallel those for ghrelin. Consequently, GOAT expression is highest in the stomach and pancreas [16, 17, 19]. Specifically, GOAT-producing cells in the stomach are mostly co-localized with ghrelin-expressing cells. As shown by immunohistochemistry, ghrelin and GOAT are co-expressed in about 70% of the gastric cells [20]. GOAT is also expressed in smaller amounts in various other tissues including the brain (pituitary and hypothalamus) [19], heart, liver, and colon [16]. Biochemically, GOAT has two critical substrates, proghrelin and short- to medium-chain fatty acids (MCFAs) that need to be conjugated as coenzyme-A (CoA) thioesters [21].

Detailed in vitro and in vivo studies aiming to understand ghrelin acylation show that GOAT acylates the prohormone proghrelin before it is cleaved to the final ghrelin peptide and released into the circulation (see Fig. 7.1 and [22, 23]). However, it also has been shown that acyl-modification of the des-acyl ghrelin peptide is possible in vitro when fatty acid CoA-esters and GOAT-containing microsomes are added to the cell-culture medium [21, 23]. While the precise mechanism and location of ghrelin acylation remain to be elucidated, studies have clearly demonstrated that GOAT cannot use free fatty acids directly from circulation for ghrelin acylation. The fatty acids need to be activated with CoA to form high-energy fatty acid CoA-thioesters in order to serve as a usable substrate for GOAT [21]. The second substrate for GOAT besides CoA-activated fatty acids is the proghrelin peptide. Intriguingly, a short sequence of only five amino acid with the structure GXSFX, where G, X, S, and F correspond to the amino acids glycine (G), any amino acid (X), serine (S), and phenylalanine (F) all with an unblocked amino terminal, respectively, is sufficient to be recognized and acylated by GOAT [21, 22]. Genome scans provide evidence that this five-amino-acid motif is only found in the amino acid sequence of ghrelin. Therefore, it can be assumed that ghrelin might be the only peptide that is acylated by GOAT.

In vitro studies show that GOAT can use CoA-activated fatty acids of various lengths ranging from acetate (C2) to tetradecanoic acid (C14) for the acyl modification of ghrelin at its critical serine-3 residue [16]. As of current knowledge most circulating ghrelin is octanoylated and to a lesser degree decanoyl-modified. These octanoyl- and decanoyl-acylated ghrelin forms are the optimal ligands for GHSR-1a because octanoyl- and decanoyl-ghrelin yield the highest GHSR-1a activation in vitro [22]. Oddly, medium-chain triglycerides (MCTs) are in general neither abundant in circulation nor rich in the regular diets consumed. The endogenous biochemical mechanisms involved in the generation of these MCFA-substrates for GOAT in the ghrelin-producing cells are still unknown. Dietary fatty acids also play an important role as GOAT substrates [24, 25] and can directly influence ghrelin acylation even though MCTs are scarce in human nutrition as compared to longer chain fatty acids. Relatively high concentrations of MCT can be found in coconut, palm oil, milk (especially human and goat milk), and dairy products. Until now it is not entirely clear to which proportion

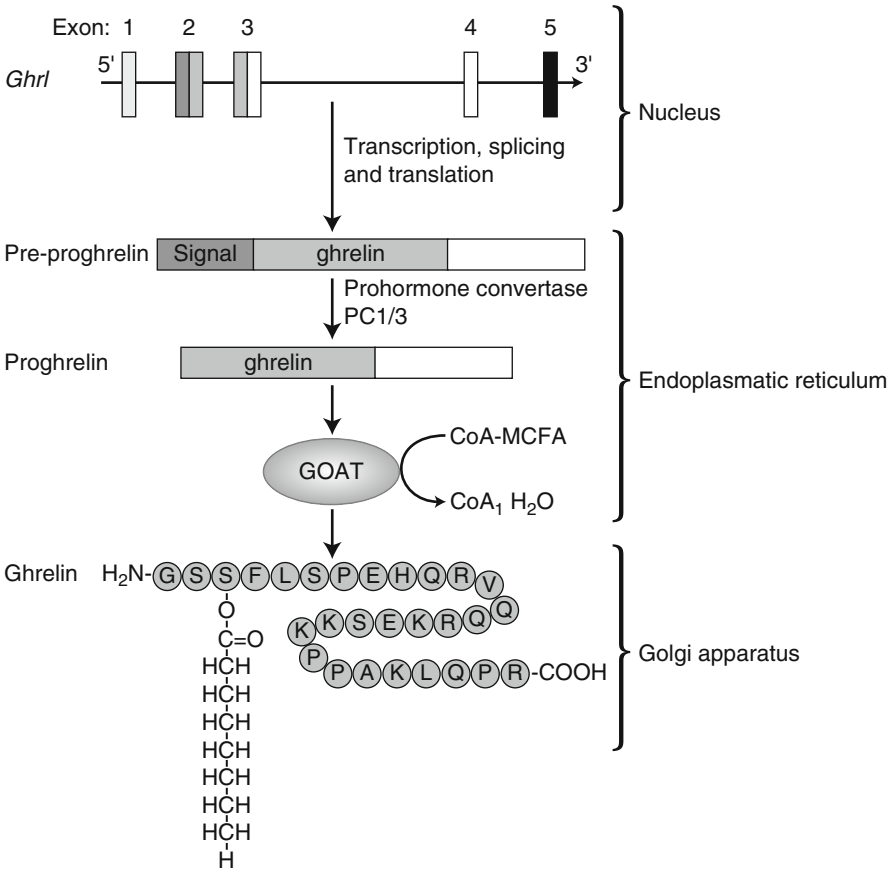

Fig. 7.1 Ghrelin biosynthesis and acylation. The 28 amino acid peptide ghrelin is encoded by exons 2 and 3 of the ghrelin gene Ghrl. In a posttranslational step pre-proghrelin is cleaved to form proghrelin by the prohormone convertase PC1/3. The GOAT that is located in the endoplasmic reticulum couples a CoA-activated medium-chain fatty acid (MCFA) to the third serine molecule of proghrelin. Acylated proghrelin is finally cleaved to form acyl-ghrelin and packaged into vesicles for secretion in the Golgi apparatus. Modified from [39]

GOAT uses dietary and endogenous fatty acids for ghrelin acylation. Further, it is has not been studied extensively if ghrelin forms that are acylated with the more abundant long-chain fatty acids such as C:16 or C:18 exist. Most recent studies comparing the in vitro selectivity of the MCFA hexanoyl- and octanoyl-CoA indicate that GOAT might actually prefer hexanoyl-CoA to octanoyl-CoA as substrates [21]. These studies highlight the importance of studying the origin and metabolism of these fatty acids in acyl ghrelin-producing cells given the knowledge that GOAT is able to use fatty acids of various chain lengths as substrates for ghrelin acylation [16].

Endogenous Regulation of GOAT Expression

GOAT activity and protein levels of GOAT are difficult to measure owing to the fact that GOAT is a membrane-bound enzyme that is likely not released into circulation. Recently, a commercially available antibody against GOAT has been developed that can be used for immunohistochemistry. This antibody is likely to provide information about local GOAT production patterns rather than GOAT quantity and activity. Therefore, the ability to precisely study the regulation of GOAT at a protein level and to describe the physiologic function of GOAT in more detail has been challenged by the technical limitations. An antibody suitable for western blot analysis that was raised against an extracellular loop of the GOAT has just been developed [26]. Studies using this antibody suggest that GOAT is released into the plasma. However, the specificity of this antibody has not been tested in GOAT-deficient mice and further characterization is required. Until reliable and highly specific anti-GOAT antibodies are made commercially available, GOAT production can be estimated by measuring GOAT mRNA levels of the GOAT gene *MBOAT4*. Further, GOAT activity can be measured indirectly by quantifying the amounts of des-acyl and acyl ghrelin in tissues and in circulation.

GOAT Expression During Development and Obesity

GOAT expression in the stomach is low in young rats and seems to increase with maturity reaching a peak in adulthood [27, 28]. This pattern is consistent with immunoreactive ghrelin-producing cells found in the stomach of rodents during development. In obese mouse models ghrelin expression in the stomach is down-regulated [29]. Gastric GOAT expression however is not changed in diet-induced obese mice compared to lean age- and gender-matched controls [19].

GOAT Expression During Feeding and Fasting

GOAT is responsible for the activation of ghrelin, the only known gut hormone that stimulates food intake. Ghrelin levels rise before meals and reach nadir after ingestion of food. Thus, GOAT expression is expected to be upregulated during fasting. GOAT gene expression studies that examine gastric *Mboat4* mRNA changes during various nutritional states however show conflicting results. One study showed that gastric expression of *Mboat4* mRNA and acyl ghrelin levels in plasma of mice are significantly increased after 24 h of fasting compared to mice fed *ad lib* [19]. Other fasting experiments in mice and rats show that gastric GOAT expression is highest when mice are fed *ad libitum* and decreases significantly with fasting for 12, 24, 36, or 48 h [24, 27]. This decreased GOAT expression during fasting is

paralleled to the unchanged acyl ghrelin concentration in blood for any duration of fasting [24]. Moreover, long-term (5 month) caloric restriction significantly decreases GOAT expression in rats [28]. Though, one study found that GOAT expression in the stomach is not changed after short-term (16 days) caloric restriction to 30% of normal food intake in rats but that gastric GOAT expression is significantly increased after 21 days of caloric restriction [27]. The discrepancy between studies may be due to differences in the length of fasting, regimen of caloric restriction, and sample collection for ghrelin measures. Some groups [19, 24, 28] sampled total stomach to measure GOAT expression, whereas other groups [27] only analyzed GOAT expression in the gastric mucosa. Compared to ghrelin, GOAT expression is relatively low even in the two major GOAT-producing tissues—stomach and pancreas. Relative to expression levels in the total stomach, GOAT expression in the gastric mucosa is lower [27]. Therefore, measurement of *Mboat4* mRNA levels in gastric mucosa may be influenced by technical factors such as detection limits of real-time PCR analysis.

GOAT Expression in Relationship with Leptin

Leptin is a major opponent of ghrelin since it activates pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript producing neurons in the hypothalamus, which leads to a reduction in food intake. Further, leptin directly regulates GHSR expression in the hypothalamus [30]. Therefore, it is of interest to investigate the interplay between leptin and GOAT. In *ob/ob* mice, a mouse model of genetic-induced obesity due to leptin deficiency, gastric GOAT expression tends to be slightly upregulated [19, 24]. Nevertheless, the direction in which leptin affects GOAT expression is not entirely clear since it has also been reported that leptin administration in fasted rats increases gastric GOAT expression [27]. The effect of leptin on gastric GOAT expression was evaluated in the study by Gonzales et al. where pharmacologic leptin treatment was given to rats that were either fasted for 48 h or fed *ad lib*. Gastric GOAT mRNA levels in rats that received vehicle administration were not different between 48 h-fasted and fed rats. Leptin administration to fed rats did not change GOAT expression. However, leptin injection in fasted rats significantly increased GOAT expression in the stomach [27]. These data indicates that GOAT gene expression may be regulated by leptin since gastric GOAT mRNA is decreased during fasting when leptin levels are low but remains unchanged when a decrease in leptin during fasting is prevented by pharmacological leptin administration.

GOAT Expression Modified by Dietary Lipids

The twofold decrease of *Mboat4* mRNA in stomach after long-term fasting does not result in lower acyl ghrelin concentrations in blood [24]. Therefore, GOAT activity

might not be a bottleneck for ghrelin acylation in *ad libitum* or fasting conditions. Given the fact that *Ghrl* expression in the stomach of mice is relatively constant during fasting [24], these data suggest that GOAT expression is possibly downregulated by the lack of available MCFAs. Surprisingly, GOAT does not seem to use lipids derived from endogenous lipolysis during fasting to acylate ghrelin. The regulation of gastric GOAT expression and acyl ghrelin plasma concentrations during short-term fasting is not entirely clear. The ability to accurately measure GOAT activity and the amount of intramembrane GOAT protein is necessary to clarify the physiological role of GOAT during fasting.

GOAT Activity Influenced by Dietary Lipids

Taken from the observation that GOAT expression is not increased during long-term fasting and the idea that GOAT might be dependent on dietary rather than endogenously derived lipids for acylation, studies were performed to identify the origin of the fatty acids used for ghrelin acylation. Feeding studies show clearly that GOAT uses fatty acids consumed with the diet to acylate ghrelin [24, 25]. Mice that are fed with a diet rich in glycerol triheptanoate, an MCT constituted of heptanoic acid (C7:0), which is not synthesized de novo in mice, produce C7-acylated ghrelin that can be found in their stomach tissue and blood [24]. Interestingly, concentrations of C7-acylated ghrelin in stomach and blood of these mice are higher than the normal C8-acylated ghrelin in mice fed a control diet. More physiologically, feeding mice a diet that is enriched with MCT produces higher amounts of octanoyl-ghrelin in blood than in control mice that are fed with standard chow [24]. These findings provide evidence that ghrelin acylation is influenced by consumed fat and that GOAT prefers dietary lipids to endogenously derived ones for ghrelin acylation. Notably, ghrelin-secreting cells in the gastrointestinal tract are the closed- and open-cell types. The open-type cells are more abundant in the duodenum [31] where fatty acids are absorbed. Thus, the open-type ghrelin cells might take up MCTs and use them directly for ghrelin acylation.

GOAT Physiology: Mutant Models and Their Energy Balance Phenotype

Currently, there is no consensus on the biological effects of des-acyl ghrelin. Some studies postulate that des-acyl ghrelin has orexigenic effects similar to those of acyl ghrelin and have antidiabetic properties opposite to that of acyl ghrelin. Phenotypical differences between GOAT-deficient GOAT-KO and Wt mice are likely to be caused by the absence of acyl ghrelin in the circulation, therefore making GOAT-KO mice an excellent tool to study des-acyl ghrelin physiology.

Energy Homeostasis of GOAT-KO Mice

To study the physiological role of GOAT on the regulation of energy and glucose homeostasis, GOAT-KO mice were developed as previously described [16, 24, 32]. GOAT-KO mice have no measurable acylated ghrelin which clearly demonstrates that GOAT is the only enzyme that acylates ghrelin [16, 24]. Total ghrelin concentration in blood, which is exclusively des-acyl ghrelin in GOAT-KO mice, is higher compared to wild-type (Wt) littermates. This finding suggests that GOAT deficiency triggers a compensatory overproduction of ghrelin in GOAT-KO mice, which leads to increased amounts of des-acyl ghrelin in tissues and circulation. Similar to ghrelin and GHSR knockout mice, GOAT-KO mice have no major changes in food intake, body weight, and body composition when fed standard chow. On chow-diet male GOAT-KO mice showed a tendency towards lower body weight and fat mass as compared to the age-matched Wt littermates while food intake was similar between the GOAT-KO and Wt mice. Similarly, female GOAT-KO mice have similar body weight, food intake, and body composition compared to Wt mice.

Ghrelin- and GHSR-deficient mice are protected against the development of diet-induced obesity when mice are exposed to high-fat diet (HFD) immediately after weaning [33, 34]. To study if GOAT deficiency ameliorates diet-induced obesity in a similar manner, GOAT-KO and Wt mice were also fed an HFD immediately after weaning. Long-term exposure to HFD leads to a significant decrease in body weight in adult male GOAT-KO mice without changing fat mass, fat-free mass, or body length. Since it could be shown that GOAT directly uses dietary lipids to acylate ghrelin (see above), male GOAT-KO and Wt mice were fed a diet enriched with MCT. In theory, this diet would not have any effect on the GOAT-KO mice because ghrelin cannot be acylated in the absence of GOAT. However, the MCT diet should enhance ghrelin acylation in Wt mice, thereby magnifying the differences between the two genotypes. Body weight of male GOAT-KO mice is significantly decreased compared to Wt mice after feeding an MCT diet. This difference is mainly due to a decrease in fat mass but not in lean mass. Interestingly, food intake is increased in GOAT-KO mice and it is therefore not responsible for the decreased body weight and fat mass of the GOAT-KO mice.

In search for possible mechanisms for the leaner phenotype of the GOAT-KO mice, energy expenditure and locomotor activity were measured using indirect calorimetry. Energy expenditure tends to be higher in male GOAT-KO mice when fed either chow or HFD but this increase is not statistically significant. On an MCT diet however, GOAT-KO mice have significantly increased energy expenditure during the light phase and overall a strong trend towards increased total energy expenditure. Fuel preference, measured by the respiratory quotient, and total locomotor activity are not different between GOAT-KO and Wt mice during MCT feeding.

Energy Homeostasis of GOAT in Ghrelin-Overproducing Transgenic Mice

In another approach to uncover the physiologic function of GOAT, transgenic (Tg) mice designed to express the human genes for ghrelin and GOAT were created [24]. As one would expect these mice have very high levels of human des-acyl and acyl (C2) ghrelin. Intriguingly, despite the high production of human des-acyl ghrelin, Tg mice do not produce human octanoyl-ghrelin. It appears that since ghrelin acylation is highly influenced by dietary lipids, under normal dietary conditions when there are insufficient MCFAs available, the selection of substrate MCFA is shifted towards the more abundant fatty acids available in the diet. Consistent with this hypothesis, high amounts of human octanoyl-ghrelin were produced when Tg mice were fed an MCT diet. This finding clearly demonstrates that the massive production of ghrelin acylation in the transgenic model is dependent on the dietary availability of MCT. Under chow feeding conditions, when Tg mice have normal blood concentrations of acyl ghrelin, Tg mice do not develop differences in body weight and body composition as compared to their Wt littermates. When fed an MCT diet however, Tg mice begin producing large amount of human octanoyl-ghrelin which leads to a 40-fold increase in blood concentrations of human acyl ghrelin as compared to Wt mice. Consequently, male Tg mice had higher body weight than the Wt littermates. This increase in body weight is largely due to an increase in fat mass based on body composition analysis.

GOAT deficiency leads to increased energy expenditure in GOAT-KO mice. As expected, Tg mice exhibit decreased energy expenditure during both the light and dark phases of the photoperiod. The respiratory quotient tends to be slightly increased in the Tg mice, which indicates less fat oxidation. Despite the very high levels of acyl ghrelin in the circulation, the food intake and locomotor activity of the Tg mice are not different from their Wt littermates.

To identify potential mechanisms that can explain the decreased energy expenditure observed in Tg mice, expressions of candidate genes were measured in muscle, liver, and brown adipose tissue in male Tg and Wt mice that were fed an MCT diet. Phosphoenolpyruvate carboxykinase (*Pepck*), a key enzyme involved in gluconeogenesis, is upregulated twofold in liver tissue of Tg mice compared to Wt mice. Expression of genes encoding the Uncoupling Protein-3 (UCP-3) and Cytochrome C, which are both important for mitochondrial respiratory chain function, are significantly reduced in quadriceps in Tg mice. The described phenotype of Tg mice disappears when mice are switched back to standard chow for only 2 weeks. This finding highlights again the importance of dietary lipids in GOAT/ghrelin system activation.

In summary, there is emerging evidence that modulation of GOAT function leads to changes in energy homeostasis. Body weight and fat mass are reduced in GOAT deficiency and increased in GOAT overproduction mouse models. Interestingly, these changes are not mediated by the alteration of food intake but rather by changes in energy expenditure, fuel partitioning, and respiratory capacity.

Relevance of GOAT and Acyl Ghrelin During Starvation

Recently another mouse model of GOAT ablation has been generated [32] to study the effects of GOAT deficiency on glucose homeostasis during *ad libitum* feeding and caloric restriction. Previous studies by Kirchner et al. showed that GOAT-KO mice have unchanged glucose tolerance compared to Wt mice when mice were fasted for 6 h and glucose was administered *intraperitoneally* [24]. Oral glucose tolerance tests performed by Zhao et al. in mice fasted for 16 h showed that GOAT-KO mice had a trend towards an improved glucose tolerance compared to Wt controls mainly due to higher insulin secretion. For the caloric restriction studies GOAT-KO and Wt mice were fed only 40% of their normal diet. As a result, blood glucose decreased in both the Wt and the GOAT-KO mice initially. However, blood glucose stabilized in the Wt group 2 days after the caloric restriction while the GOAT-KO mice were unable to preserve blood glucose after they lost 75% of their body fat and became hypoglycemic and morbid bound after 7 days. Furthermore, GOAT-KO mice had improved glucose tolerance during an oral GTT 5 days after caloric restriction begun. The hypoglycemia in GOAT-KO mice was not caused by increased insulin secretion or by glucagon deficiency. In addition, expression of genes involved in gluconeogenesis and lipolysis in the liver was normal.

Growth hormone (GH) contributes to maintain physiological blood glucose levels during states of caloric restriction. Consequently, caloric restricted Wt and GOAT-KO mice have higher levels of GH compared to mice fed *ad libitum*. Nevertheless, calorically restricted GOAT-KO mice have twofold lower levels of GH as compared to Wt mice. This finding indicates that GOAT-KO mice develop a relative but not absolute GH deficiency due to the lack of ghrelin-induced GH secretion during caloric restriction. Replacement therapy of either ghrelin or GH in GOAT-KO mice during caloric restriction prevents the development of severe hypoglycemia, which may be mediated by ghrelin and GH. Nevertheless, it should be notated that the severe hypoglycemic phenotype of calorically restricted GOAT-KO mice is only apparent when mice reach a body fat of <2% of their total body weight and when blood glucose is measured after 22 h of fasting. Blood glucose was low at earlier time points (e.g., after 14 h of fasting) but was still within the normal range in GOAT-KO mice.

In summary, available data suggest that acyl ghrelin may play a role in physiological adaptation to fasting and caloric restriction via its effect on stimulating GH secretion and glucose homeostasis. Under normal conditions, the organism is able to maintain normal GH secretion that is required for growth even in the absence of GOAT, ghrelin, or GHSR [24, 33, 34]. Thus, acyl ghrelin and GOAT appear to be only necessary to enhance GH secretion in times when extra GH is required such as severe calorie restriction. More studies are needed to confirm such findings and provide better understanding of the role of the ghrelin/GOAT system in varying nutritional states.

A New Role for the GOAT–Ghrelin System

Gastric GOAT, ghrelin expression, and circulating acyl ghrelin levels are not increased during long-term starvation or caloric restriction in humans [8] or rodents [24, 27]. Therefore, it does not seem likely that either GOAT or ghrelin expression is essential for the generation of a hunger signal that indicates an empty stomach. However, ghrelin acylation and thereby activation seem to be influenced substantially by nutrient, particularly fatty acid composition, in the diet suggesting that ghrelin might be a gastric lipid sensor or nutrikine that signals the brain for the abundance of calories [24]. This proposed new GOAT–ghrelin model viewing ghrelin as a nutrikine is shown schematically in Fig. 7.2. The substrate for GOAT-mediated acylation is limited during prolonged fasting when dietary lipids are scarce. Endogenously derived free fatty acids, which are abundant during fasting, are not used to compensate for the lack of dietary fatty acids (Fig. 7.2a). However, they are used, to a less extent, to maintain basal ghrelin signaling, since blood concentrations of acyl ghrelin have never been reported to be zero, even after prolonged fasting. Downregulation of *Mboat4* transcript and shortage of dietary fatty acids lead to a relative overproduction of des-acyl ghrelin, which cannot bind to or activate GHSR. It is speculated that the body may utilize different fatty acid resources for ghrelin acylation during different phases of digestion. In the preprandial state during normal food intake (Fig. 7.2b), the source of fatty acids used for ghrelin acylation could be either from endogenous lipolysis or dietary lipids that remained in the stomach from a previous meal. Further, circulating acyl ghrelin in between meals could come from preexisting ghrelin substrates. After a meal, MCTs are readily absorbed and used for ghrelin acylation (Fig. 7.2c). The MCFAs coupled to ghrelin enter cells of the CNS via GHSR. The fate of the MCFA after the acyl ghrelin–GHSR complex is internalized and remains unknown but may be utilized for hypothalamic fatty acid metabolism [35]. The proposed new lipid-sensing role of GOAT–ghrelin seems logical when the main functions of ghrelin are lipogenesis and induction of GH release, two features that seem to be counterintuitive during hunger. It would make more sense for an organism to increase cell proliferation and fat storage when incoming nutrient is readily available. This theory of the novel function of the GOAT–ghrelin system remains to be tested. Follow-up studies that provide better insight into fatty acid uptake by the ghrelin-producing cells and the destination of the fatty acids after absorption are needed to help understand and more efficiently explore the mechanistic details of ghrelin acylation by GOAT.

Therapeutic Potentials of Pharmacological Ghrelin and GOAT Modulation

When ghrelin was discovered and regarded as a meal initiation factor, antagonists for the ghrelin receptor became an interesting therapeutic target for the treatment of obesity and type 2 diabetes. One of the obstacles of designing a ghrelin receptor antagonist is that it needs to pass the blood–brain-barrier to efficiently decrease food intake and

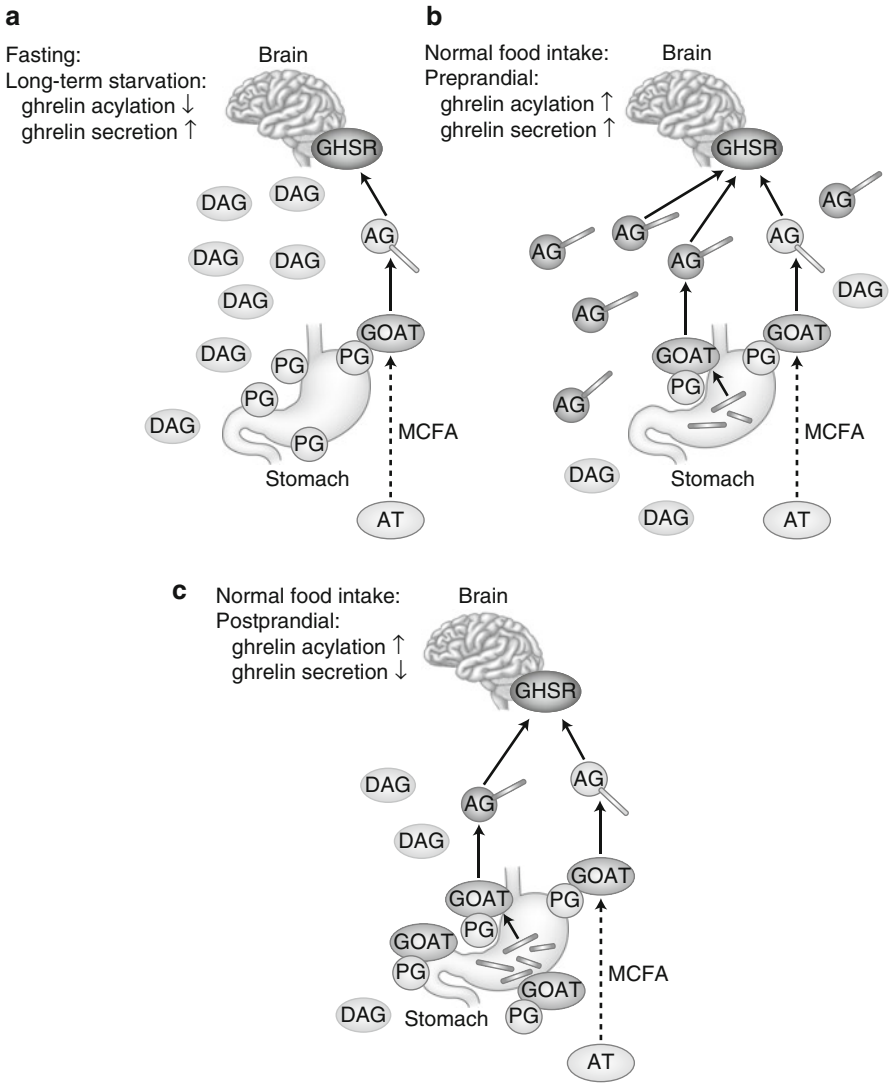

Fig. 7.2 Ghrelin as a nutrikine that signals the brain of abundance of dietary lipids. (**a**) Basal ghrelin signaling comprises dietary and endogenously derived medium-chain fatty acids (MCFA) that are coupled to ghrelin via GOAT to form the bioactive acyl ghrelin (AG). (**b**) During fasting, ghrelin acylation is reduced due to a lack of dietary lipids and the downregulation of gastric GOAT gene expression. Only a small amount of adipose tissue (AT)-derived fatty acids are used to maintain the low ghrelin signaling during this period. Gene expression of ghrelin is not downregulated by fasting, which results in relative overproduction of des-acyl ghrelin (DAG). (**c**) Ingested MCFA is directly used by GOAT to synthesize acylate ghrelin. The high blood concentration of AG leads to enhanced GHSR signaling that possibly signals the brain for a calorie-rich environment

body weight, at least in rodent models [36, 37]. Therefore, modifying GOAT became an attractive alternative approach to alter ghrelin action, especially when ghrelin seems to be the only peptide substrate for GOAT. The loss- and gain-of-GOAT-function

mouse models provide solid evidence that GOAT plays a role in the regulation of body weight and adiposity [24]. Thus, GOAT modulators may be effective new antiobesity drugs or anti-cachexia therapeutics. To test this notion, GOAT-inhibiting molecules have been developed recently [22, 38]. Yang et al. designed GOAT-inhibiting peptides using a newly developed biochemical assay to study GOAT activity in vitro [22]. This assay uses membranes from insect cells that were infected with a baculovirus that produce mouse GOAT. With this assay, GOAT activity was found to be enhanced by the presence of long-chain fatty acyl-CoAs (14–18 carbons). Secondly, the substrate recognition sequence of GOAT was identified as glycine-1, serine-3, and phenylalanine-4. These two findings were crucial for the development of potential GOAT agonists and antagonists since replacement of one of these amino acids leads to inhibition of GOAT. Subsequent experiments by these investigators demonstrated that potent GOAT inhibition could be achieved when the ghrelin-like pentapeptide that corresponds to the GOAT recognition sequence is amidated at the C-terminus. Interestingly, this amidated ghrelin-like pentapeptide is able to be octanoylated by GOAT and the inhibitory effects on GOAT activity are likely to be due to end-product inhibition. Encouraged by these findings, Yang and colleagues designed synthetic pentapeptides with various amino acid substitutions and acyl side chains in search for a potent GOAT inhibitor [22]. The most efficient inhibition of GOAT activity was achieved when serine-3 was replaced by octanoylated (S)-2,3-diaminopropionc acid (Dap) on the above-described amidated ghrelin-like pentapeptide. Importantly, the octanoyl-residue was linked to Dap through an amide bond instead of the typical ester bond as seen with the native ghrelin.

More recently another GOAT inhibitor named GO-CoA-Tat was developed and shows impressive effects on energy and glucose homeostasis in vivo [38]. The bisubstrate analog GO-CoA-Tat was designed based on the knowledge that GOAT uses the two substrates octanoyl-CoA and pro-ghrelin to form octanoyl-ghrelin through a ternary complex mechanism. The GOAT-inhibiting action of GO-CoA-Tat was achieved by linking the two GOAT substrates with a non-cleavable bridge so that the binding energies of the individual ligands were combined without the entropic loss associated with forming the physiological ternary complex. To study the GOAT-inhibitory effects of GO-CoA-Tat in vitro, cell models were developed to consistently express GOAT and pre-proghrelin. GO-CoA-Tat inhibits the production of acyl ghrelin with maximal efficiency 24 h after treatment. This delay in inhibition of acyl ghrelin production was thought to be due to preformed acyl ghrelin stores within the cells. To test this hypothesis, the effects of GO-CoA-Tat on acyl ghrelin production were tested in vitro on recombinant microsomal GOAT. Using this model, nearly complete GOAT inhibition was achieved by GO-CoA-Tat treatment only after 5 min. Consequently it can be assumed that significant amounts of preformed acyl ghrelin exists and is likely to be stored in intracellular reservoirs. Furthermore, the authors tested the effect of GOAT inhibition in vivo by injecting 11 μmol/kg (40 mg/kg) GO-CoA-Tat *i.p.* into wild-type mice. GO-CoA-Tat treatment successfully reduced acyl ghrelin concentrations in serum with maximal inhibition 6 h after injection. Importantly, GO-CoA-Tat had no effect on serum concentration of des-acyl ghrelin. The strong inhibition of acyl ghrelin production led to significant reduction in body

weight and fat mass without changes in food intake in mice that received chronic treatment of GO-CoA-Tat as compared to mice treated with placebo. No toxic effects of GO-CoA-Tat treatment on liver, renal, pancreas, or bone marrow were observed in the study. Moreover, GO-CoA-Tat is very likely to have GOAT-specific effects since treatment with GO-CoA-Tat in ghrelin KO mice did not change body weight and fat mass in comparison with ghrelin KO mice that were treated with control substance. Interestingly, blood analysis showed that Wt mice treated with GO-CoA-Tat had lower blood glucose and lower IGF-1 levels compared to mice treated with control substance. To further investigate the effect of pharmacological GOAT inhibition on glucose homeostasis, mice were pretreated with GO-CoA-Tat and then underwent a glucose tolerance test 24 h after the GO-CoA-Tat injection. GO-CoA-Tat-treated mice had higher insulin secretion and lower blood glucose in response to the glucose administration as compared to control mice. The beneficial effect of GO-CoA-Tat on glucose tolerance appears to be specific for GOAT inhibition since glucose tolerance and insulin secretion were not altered in ghrelin-knockout mice treated with GO-CoA-Tat. Similar to the Wt mouse studies, human islets that were pretreated with GO-CoA-Tat showed significantly increased insulin production compared to the control. To identify possible mechanisms for the beneficial effects of GOAT inhibition on glucose tolerance, pancreatic islets were isolated from Wt mice and were treated with GO-CoA-Tat. Real-time PCR analysis revealed that expression of uncoupling protein 2 (UCP2), which is known to regulate insulin secretion, was 20-fold lower in islets from mice that were treated with GO-CoA-Tat. These findings suggest that GOAT inhibitors may act directly on pancreatic islet cells to stimulate insulin secretion by suppressing UCP2 expression.

We are only at the infancy stage of our journey to discover the true faces of GOAT. There is emerging evidence that GOAT plays a role in regulating energy balance and glucose homeostasis likely by governing the ghrelin acylation process. The relevance of GOAT and acyl ghrelin during periods of starvation remains to be defined. The theory that GOAT functions as a "nutrikine" to signal the CNS for nutrient abundance also requires further testing and confirmation. The development of GOAT-specific inhibitors provides scientists with an important tool to unlock the mystery of the true function of the ghrelin–GOAT system in physiology. Moreover, the metabolic benefits seen with pharmacological GOAT inhibition in vivo raise hope that GOAT may be a more feasible and effective target than the ghrelin receptor or ghrelin isoforms for the treatment of metabolic disorders such as obesity and type 2 diabetes.

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Chapter 8
Ghrelin Regulation of Sleep, Circadian Clock, and Body Temperature

Éva Szentirmai and Levente Kapás

Abstract The regulation of sleep, body temperature, and metabolism is intertwined on functional, structural, and behavioral levels. The hypothalamus emerges as a key brain region that coordinates these functions by integrating central and peripheral signals. Increasing evidence suggests that products of the preproghrelin gene provide important contribution to this integrative function. In this review, we present evidence from human and animal studies supporting the role of ghrelin in the regulation of (1) sleep–wake activity, (2) circadian rhythms, and (3) metabolism and body temperature. Central ghrelinergic mechanisms—as part of the hypothalamic ghrelin–orexin–neuropeptide Y circuit—play a role in promoting wakefulness and feeding. Ghrelin modulates the activity of the suprachiasmatic nucleus but it is not a crucial component of or a key input signal to the food-entrainable oscillator. Products of the preproghrelin gene, particularly obestatin, are involved in maintaining normal body temperature and metabolism under conditions when increased metabolic heat production is required. Our understanding of the physiological role of the preproghrelin gene products expanded remarkably in the last decade, yet, full comprehension of their role in the regulation of vigilance, circadian clocks, and body temperature remains incomplete.

É. Szentirmai, M.D., Ph.D. (✉) • L. Kapás, M.D., Ph.D.
WWAMI Medical Education Program, Washington State University,
412 E. Spokane Falls Blvd, Spokane, WA 99202, USA

Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology,
Washington State University, Pullman, WA 99202, USA
e-mail: eszentirmai@wsu.edu

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Introduction

The relationship between the regulation of sleep, metabolism, and body temperature has long been acknowledged. Sleep is accompanied by characteristic changes in metabolism and body temperature. Conversely, changes in metabolism and the overall nutritional state of the organism affect sleep. Historically, causative relationships between vigilance and metabolism were simply treated as metabolic changes being passive consequences of shifts in vigilance. While this remains undisputed, recent studies focus more on the complexities in the interaction of sleep and metabolism. The regulation of vigilance and metabolism share signaling mechanisms and some metabolic responses linked to vigilance shifts are not caused by sleep per se but are independent manifestations of the action of a common regulatory mechanism. Furthermore, the regulation of sleep, feeding, and metabolism overlaps on the structural level. Several hypothalamic areas, such as the suprachiasmatic nucleus (SCN), lateral hypothalamus (LH), and ventromedial hypothalamic nucleus (VMH), are implicated in the regulation of both sleep and metabolism/food intake [1]. These structures express receptors for a variety of hormones of the gastrointestinal tract and adipose tissue. These hormonal signals can modulate sleep and metabolism independent of one another and may play a role in aligning vigilance with the current metabolic state of the body. A growing body of evidence suggests that the products of the preproghrelin (Ppg) gene may serve these functions.

Ghrelin and Sleep

The Effects of Growth Hormone Secretagogues on Sleep in Humans

The presence of growth hormone secretagogue receptors (GHS-R) was initially recognized by the ability of certain synthetic peptides, called growth hormone secretagogues (GHSs), to stimulate growth hormone (GH) secretion and their binding to pituitary membranes (reviewed in [2]). Even before ghrelin was identified as the endogenous ligand for the GHS-R1a (herein we refer to this receptor as the ghrelin receptor), there were several reports on the effects of GHSs on sleep. The initial interest in GHSs in sleep regulation stemmed from the recognition of the close relationship between sleep and somatotropic axis. By the late 1990s, growth hormone-releasing hormone (GHRH) had been well-characterized as an endogenous sleep-promoting hormone. Its non-rapid-eye-movement sleep (NREMS)-inducing actions had been documented in rats, rabbits, mice, and humans (reviewed in [3]). The findings that GHSs mimic the effects of GHRH on GH secretion directed the attention to other possible similarities between GHRH and GHSs, including potential similarities in their effects on sleep. A series of human experiments were

initiated, mainly driven by the fact that some of the GHSs are active when administered orally, thereby suggesting their use in sleep medicine should they possess GHRH-like sleep-promoting effects. Four GHSs were tested in humans. Growth hormone-releasing peptide-6 (GHRP-6) and hexarelin were studied at the Max-Plank Institute in Munich and growth hormone-releasing peptide-2 (GHRP-2) and MK-677 at the Université Libre de Bruxelles. Unfortunately, a clear view regarding the effects of GHSs on human sleep failed to emerge from these otherwise well-designed and executed experiments. As discussed below, various GHSs have divergent, even opposite effects on sleep and the effects of a given GHS may vary according to the route of administration in humans.

The first ghrelin receptor agonist studied for sleep effects in humans was GHRP-6. Pulsatile administration of GHRP-6 to young men by using four intravenous (iv) bolus injections did not affect total sleep time, sleep latency, or other sleep-related electroencephalographic (EEG) variables; only a modest increase in stage 2 sleep was observed. Plasma GH and cortisol levels, however, significantly increased after GHRP-6 treatment indicating that physiologically relevant doses were tested [4]. In a subsequent study, the same research group took advantage of the ability of GHRP-6 to be absorbed through mucous membranes after *per os*, sublingual, or intranasal administration. In contrast to the weak sleep-inducing effects after iv injection, *per os* administration of GHRP-6 to young males before bedtime significantly decreased total sleep, increased sleep latency, and suppressed stage 2 NREMS in the second half of the night while the sublingual or intranasal administration had no effect [5]. Sleep continuity, sleep architecture, and rapid-eye-movement sleep (REMS) variables remained unchanged after each route of administration. Significant increases in plasma GH and cortisol occurred; these hormone responses after intranasal GHRP-6 administration were comparable in magnitude to those occurring after pulsatile iv injection of the GHS.

Another orally active GHS, MK-677, increased the duration of stage 4 sleep by 50% and REMS by 20% in young male subjects after one week of treatment. In older adults, treatment with MK-677 was associated with a ~50% increase in REM sleep and a significant decrease in REM latency [6]. In a subsequent experiment by the same research group, iv bolus injection of GHRP-2 failed to have any effect on sleep EEG or on the amount of slow-wave sleep (SWS) in young healthy men when given after the third REMS period during the night [7]. In light of the negative findings of the latter study, the authors concluded that the multiple complex mechanisms may be involved in the sleep-promoting activities of MK-677 which are likely independent of the stimulatory effects on the ghrelin receptors.

In the most recent study in the series of GHS experiments in humans, hexarelin was tested, the most potent known synthetic peptide agonist of the ghrelin receptors in terms of the stimulation of GH release. Pulsatile iv injection of hexarelin to young males led to decreased deep, stage 4 sleep during the first half of the night and suppressed EEG delta power during NREMS (a measure used for characterizing the intensity-depth of NREMS) across the entire night. REMS and sleep continuity were not affected [8]. In conclusion, various GHSs had different actions on human sleep.

The Effects of Ghrelin on Sleep in Humans

Human studies with ghrelin began at the Max-Plank Institute in Munich shortly after its identification as the endogenous GHS receptor agonist. In an elegant series of experiments, ghrelin was tested in young and elder men and women using various administration schedules. In these studies, sleep recordings were complemented with simultaneous measurements of plasma GH and cortisol levels. In the first study, four hourly iv bolus injections of ghrelin were given during the late evening hours between 2200 h and 0100 h to young healthy males [9]. This injection schedule of ghrelin enhanced SWS (particularly stage 4) during the first half of the night, and increased EEG delta activity in the second half of the night. REMS was not affected and other measures of sleep continuity and sleep architecture remained unchanged. In these studies, ghrelin also elevated plasma GH and prolactin levels during the first and cortisol levels during the second half of the night. These findings confirmed prior studies showing the stimulatory effects of ghrelin on the somatotropic and hypothalamic–pituitary–adrenal (HPA) axes in humans [10].

Components of the somatotropic and HPA axes are implicated in sleep regulation. GHRH stimulates sleep while corticotropin-releasing hormone (CRH) suppresses sleep both in humans and various other species (reviewed in [11]). Since ghrelin stimulates both the somatotropic and HPA axes, it was of an interest to investigate the interactions of ghrelin with GHRH and CRH in sleep regulation [12]. In this study, the same treatment protocol was followed as in the study described in the previous paragraph. Placebo or ghrelin alone or in combination with CRH or GHRH was injected during the first part of the night to young men. In contrast to the previous findings, ghrelin itself had no effect on any of the sleep parameters during the first half of the night while stage 2 NREMS was increased during the second half of the night by ~20 min. Ghrelin treatment significantly increased both GH and cortisol levels during the injection period. Coadministration of GHRH or CRH with ghrelin did not modify the sleep effects of ghrelin but potentiated its GH- and cortisol-stimulating effects, respectively.

The timing of the ghrelin treatment and gender are two major factors that seem to determine the effects of ghrelin on human sleep. When sequential injections of ghrelin are performed in the early morning hours instead of the first half of the night, ghrelin loses its effects on sleep and EEG while the GH- and cortisol-stimulating effects persist [13]. Ghrelin does not appear to have any effect on sleep in females. When it is injected late night to young women [14] or postmenopausal elderly women [15], ghrelin failed to affect any of the sleep parameters while it stimulated plasma GH and cortisol levels similarly to that seen in elderly men. In elderly men, however, the same treatment increased deep, stage 4 NREMS during the first part of the night and stage 2 NREMS during the second half [15]. Recently, ghrelin was tested in patients with major depression [16]. In depressed men, ghrelin significantly reduced the time spent awake in the second part of the night without affecting any parameters of NREMS and REMS. In depressed women, however, waking was not altered but the amount of REMS was decreased. These latter findings

differ from those in healthy young and elderly women whose sleep was not altered by ghrelin [14, 15]. GH and cortisol responses to ghrelin were similar to those seen in healthy subjects.

The emerging picture from the human experiments is that when ghrelin is administered in iv pulses during the first part of the night it induces slight increases in sleep in young men. In healthy women, young or elder, ghrelin has no effects on sleep parameters. Further, if ghrelin is administered during the second part of the night, it loses its modest sleep-promoting activity even in young males. Ghrelin consistently stimulates plasma GH and cortisol levels in each age group and gender irrespective of the timing of administration.

Animal Studies

The role of ghrelin in sleep regulation has been studied in rats and transgenic—preproghrelin knockout (Ppg KO, originally named as ghrelin –/– mice) and ghrelin receptor KO—mice. Results from these studies strongly suggest that central ghrelin signaling is a key component of the arousal system. Two main lines of evidence support this notion. One, systemic, intracerebroventricular (icv) or intrahypothalamic administration of exogenous ghrelin increases wakefulness in rats and, two, in the absence of ghrelin signaling the responsiveness of wake-inducing mechanisms to major arousal-promoting stimuli is greatly impaired.

The Effects of Ghrelin on Sleep in Rats

The first rat sleep study on the effects of ghrelin used sequential iv injections. The first ghrelin injection was done 1.5 h before the end of the light period and two subsequent treatments were given during the early dark phase [17]. Ghrelin increased wakefulness and decreased NREMS for 30 min immediately after all three injections. REMS was decreased after the injections of ghrelin during the dark phase. Consistent with these wake-promoting effects, systemic [18], icv [19–21], intra-ventral tegmental area (VTA), or intra-laterodorsal tegmental area (LDT) [22] injections of ghrelin increase locomotor activity.

Similar to the effects of systemic ghrelin treatment, light- or dark-onset icv administration of ghrelin also induced significant dose-dependent increases in wakefulness (Fig. 8.1) with the concomitant suppression of both NREMS and REMS [23]. Increased wakefulness was accompanied by signs of behavioral activation in the first postinjection hour including increased locomotor activity, eating, drinking, grooming, and exploration. The first feeding bout occurred 10 min after the injection and eating continued throughout the first hour of the light period. Feeding behavior per se, however, is not responsible for the wake-promoting effect of ghrelin since it continued to be present when animals did not have access to food.

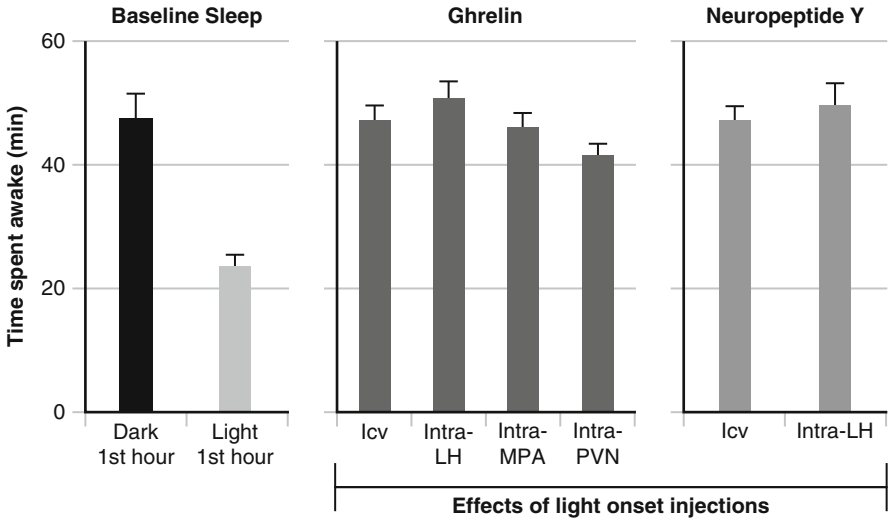

Fig. 8.1 The amount of time spent in wakefulness in the first hour of the dark and light periods under baseline conditions and after intracerebroventricular (ICV) or intrahypothalamic administrations of ghrelin and neuropeptide Y (NPY) in the first hour of the light period in rats. Under baseline conditions, rats spend most of the first hour of the night awake, while they mostly sleep in the first hour of the light phase. Injection of ghrelin or NPY at the beginning of the light induced increases in wakefulness to the level seen normally at the beginning of the dark phase. *LH* lateral hypothalamus, *MPA* medial preoptic area, *PVN* paraventricular nucleus

In the brain, ghrelin receptors are widely expressed in the hypothalamus as well as in various extrahypothalamic structures such as the hippocampus and the mesolimbic system [24–26]. Microinjection studies were performed to identify the central target(s) where ghrelin induces wakefulness. One of the likely targets considered was the LH. The LH plays a key role in the regulation of vigilance [27] and feeding [28]. Ghrelin receptors are expressed [29, 30] and ghrelinergic axon terminals are present [29, 31, 32] in the LH and local administration of ghrelin stimulates eating [33, 34]. Ghrelin microinjections into the LH induced a dose-dependent increase in wakefulness (Fig. 8.1) and suppression of NREMS and REMS lasting for 2 h and, simultaneously, stimulated feeding in rats [35].

Similar to the effects of ghrelin, intra-LH microinjections of neuropeptide Y (NPY) (Fig. 8.1 and [36]) and icv administration of orexin also induce wakefulness [37] and eating [38]. The resemblant effects of these neuropeptides are likely more than coincidence. Ghrelin, NPY, and orexin neurons in the hypothalamus form a well-defined circuit. Ghrelin-producing neurons are present in the arcuate nucleus (ARC), LH, paraventricular nucleus (PVN), hypothalamic area adjacent to the ARC, VMH, and dorsomedial hypothalamic nucleus (DMH) [32, 39]. Ghrelin neurons receive inputs from NPY-, orexin-, and pro-opiomelanocortin (POMC)-containing axon terminals in the ARC. Ghrelin-containing axon terminals are found in the LH and ARC where they synapse with orexinergic [31] and

NPYergic [32] neurons, respectively. Ghrelin neurons also project to the PVN where they stimulate CRH cells by promoting the release of NPY [32]. NPY cells in the ARC express ghrelin [40] and leptin [41] receptors; circulating ghrelin and leptin modulate the activity of this circuit by, respectively, stimulating or inhibiting NPY neurons in the ARC (reviewed in [42]). NPYergic neurons originating from the ARC synapse on orexin-positive cells in the LH which, in turn, project back to NPY cells [43].

Orexinergic neurons are located in the LH. The close relationship between ghrelin and orexin in the LH is well documented. Ghrelin-containing axon terminals make direct synaptic contacts on orexin neurons [31] and icv or local microinjection of ghrelin activates these cells [31, 33, 44, 45]. Increased feeding in response to ghrelin application into the LH is, at least in part, mediated by orexin [31]. It is possible that orexin mechanisms also play a role in ghrelin-induced arousal responses because orexin is a key component in arousal mechanisms (reviewed in [46]). Orexin-expressing neurons from the LH send dense and diffuse projections to the cerebral cortex as well as they innervate forebrain and brainstem structures that are implicated in arousal [47]. Icv injection of orexin A in rodents reduces REMS and NREMS, and increases wakefulness [37, 48]. Microinjections of orexin into the locus ceruleus, tuberomammillary nucleus, LDT, and lateral preoptic area have similar wake-promoting effects [49–52]. Direct and selective optogenetic photostimulation of LH orexin neurons in freely moving mice facilitates transition from sleep to wakefulness [53]. Orexinergic neurons discharge during active wakefulness while they are silent during SWS [54]. The sleep disorder narcolepsy is linked to the lack of orexin and/or orexin receptors [55, 56]. We posit that orexinergic projections from the LH constitute a major output for ghrelin mechanisms in the hypothalamus. It is possible, however, that the interaction between ghrelin and orexin in regulating vigilance is not limited to the LH. For example, in the pedunculopontine tegmentum, a structure involved in the maintenance of wakefulness and REMS (reviewed in [57]), orexin and ghrelin activate the same neurons in an additive manner [58].

Increased activity of the ghrelin–NPY–orexin circuit leads to the stimulation, whereas decreased activity causes the suppression of feeding. We propose that the same circuit is shared by the arousal system and its activation leads to wakefulness. The activity of the circuit is stimulated by metabolic signals during negative energy states such as fasting. In fact, short-term fasting leads to increased wakefulness [59]. This response is blunted in mice with the genetic disruption of ghrelin signaling ([59] and also see below). Increased feeding activity and long periods of wakefulness are characteristic occurrences during the first part of the dark phase in nocturnal rodents. We posit that the hypothalamic orexin–ghrelin–NPY circuit integrates metabolic, circadian, and possibly homeostatic sleep signals as well as signals arising from the external environment. At the beginning of the behaviorally active period, increased activity of the circuit is manifested as a coordinated behavioral syndrome, called the dark-onset syndrome in rats. This syndrome entails increased arousal and feeding, the two parallel manifestations of the activation of the same hypothalamic circuit.

In addition to the LH, other potential wake-inducing targets for ghrelin to induce wakefulness include the medial preoptic area (MPA) and the PVN. Ghrelin microinjection into these nuclei also increases the amount of wakefulness (Fig. 8.1 and [35]) and feeding [34, 35] in rats. The importance of the MPA in the hypothalamic sleep-regulating system [27] and the PVN in arousal, autonomic, and behavioral responses to stressors [60] is well documented. It is possible that ghrelin's wakefulness- and feeding-promoting effects are mediated through the release of nitric oxide (NO) in the MPA. NO-producing mechanisms are implicated in the regulation of sleep [61] and feeding [62]. Microinjection of a NO-donor into the MPA increases arousal [63] and the feeding-stimulatory actions of ghrelin are NO dependent [62, 64]. The PVN appears to be the least sensitive among the hypothalamic sites tested for ghrelin's sleep-modulating effect [35]. It is possible that ghrelin's actions in the PVN are mediated, in part, through the activation of the HPA axis. Ghrelin facilitates CRH release in the PVN through stimulating GABA release from NPY neurons [32]. CRH signaling is an integral component of the arousal system [11].

The Effects of Obestatin on Sleep

In addition to ghrelin, the ghrelin gene also codes for obestatin [65] and other alternative mRNA transcripts; the function of the corresponding peptides remains unidentified [66]. Presently, the gene is commonly referred to as Ppg gene indicating that it gives rise to multiple biologically active products. While the physiological role of obestatin is still debated, some results suggest that its effects on feeding may be the opposite of ghrelin's [65, 67–69]. Interestingly, such a dichotomy is also apparent in the effects of the two peptides on sleep. While ghrelin has strong wake-promoting activities, obestatin injection into the lateral cerebral ventricle of rats induces a significant increase (~58%) in NREMS amount, shortens sleep latency, and suppresses EEG slow-wave activity in the first hour after the injection [70]. The increase in NREMS time is due to an increase in the number of NREMS episodes. Systemic injection of obestatin does not affect sleep.

Sleep in Ppg and Ghrelin Receptor KO Mice

Despite the potent food intake-stimulating effect of ghrelin, the congenital deletion of the Ppg gene in mice results in normal phenotype and feeding pattern, normal body weight, growth rate, body composition, and food intake [71]. Also, while exogenous administration of ghrelin causes robust increases in wakefulness, Ppg KO animals which lack all peptide products of the Ppg gene, including obestatin, do not show major change in their sleep–wake pattern under normal conditions at thermoneutral ambient temperature [72]. However, compared to wild-type (WT) mice, NREMS in the Ppg KO animals is more fragmented with higher number but shorter NREMS episodes. Interestingly, other transgenic mouse strains lacking key

components of arousal-promoting mechanisms such as orexin [73] or histamine [74] also exhibit relatively normal sleep duration yet fragmented sleep architecture. Sleep deprivation for the last 6 h of the light period in Ppg KO mice induces normal rebound sleep suggesting that KO animals possess adequate homeostatic sleep mechanisms [72].

Genetic deletion is widely used to study biological functions of genes in mice; negative findings, however, have to be viewed with caution. The consequence of a single gene disruption on complex biological phenomena, such as feeding behavior or vigilance, is often ambiguous because of the development of compensatory means or the activation of preexisting, redundant mechanisms. The lack of the effect of single gene deletion on behavior only suggests that either the gene product has no critical role in a given function or, if it has, its absence is compensated for. These compensatory mechanisms are likely to emerge and strengthen during brain development in response to stimuli that the animal regularly encounters. Compensation, however, may not be sufficient to maintain normal behavior in response to stimuli that are not present during brain development. The behavioral response of the adult may be defective to such stimuli, thus unveiling the function of the gene product.

In recent experiments, ghrelin receptor KO mice were challenged with novel environment and fasting, two stimuli/conditions unlikely to be encountered during intrauterine development or the preweaning period in the nest. In WT animals, exposure to a novel environment or 24-h fasting elicits robust arousal responses. Wakefulness is greatly increased for 2 h in a new environment and for ~12 h during the night of food deprivation. In the KO mice, arousal responses to novel environment were reduced by about 50% and responses to fasting were completely abolished [75]. These findings are consistent with reports that ghrelin enhances exploratory activity in a novel environment [19, 21] and that the alcohol-induced locomotor stimulation is attenuated in ghrelin receptor KO [76] and Ppg KO [77] mice. Together, they strongly support a role for ghrelin signaling in arousal mechanisms.

Endogenous Ghrelin Levels and Sleep–Wake Activity

In humans and rodents the major factor that influences plasma ghrelin levels is the feeding status; fasting stimulates, while feeding suppresses, plasma ghrelin. In addition, sleep- and circadian-related factors independently affect plasma ghrelin levels. In rats, there are distinct diurnal rhythms in plasma ghrelin. During the light phase, when rats spend most of the time sleeping and eating is minimal, ghrelin levels increase reaching the maximum in the middle of the day; thereafter ghrelin gradually decreases and stays low during the night. Sleep deprivation for the first 5 h of the light phase significantly increases plasma and hypothalamic ghrelin levels in rats [78]. In humans, circulating ghrelin levels are high during the night in sleeping, thus fasting, subjects and rise further in the morning hours, before breakfast. During the day, ghrelin levels rise sharply before each mealtime and decline precipitously within 60 min after eating [79]. In healthy male volunteers, ghrelin levels increased

during the early part of the night and decreased in the morning [80]. The nocturnal increase is blunted if the subjects stay awake for the whole night but remain in bed. Sleep deprivation for one night in healthy young subjects advances the ghrelin plasma level maximum to an earlier part of the day [81].

Ghrelin and the Biological Clocks

Energy homeostasis, feeding, and sleep–wake activity display a robust 24-h circadian rhythm driven by biological clocks. The clock activities are entrained by environmental signals, called Zeitgebers, to synchronize the spontaneous rhythm of the clock(s) to the 24-h solar cycle. The "master" clock in mammals is the SCN. The main Zeitgeber for the SCN is the light, but non-photic cues can also modulate SCN activity, phase shift circadian rhythms, or interfere with the phase-shifting effects of light. Some of these non-photic cues are related to metabolism, e.g., food availability, calorie restriction, or change in glucose availability (reviewed in [82]). In addition to the SCN, these metabolic cues also act on another independent clock, called the food-entrainable oscillator (FEO). The secretion of many gastrointestinal hormones, including that of ghrelin, is phase-locked with the feeding/nutritional status of the body making them good candidates for relaying metabolic information to the biological clocks.

Ghrelin and the Suprachiasmatic Nucleus

The possible role of ghrelin in SCN signaling is supported by the presence of ghrelin receptors in the SCN [24, 26, 29] and the expression of ghrelin-like immunoreactivity in SCN neuronal processes in rats [32] and humans [83]. If ghrelin is applied to cultured hypothalamic slices, it induces a ~3-h phase advance in the electrical activity rhythm of SCN neurons [84]. Similar phase advances occur in response to ghrelin administration in the rhythm of Period2::Luciferase expression in SCN slices from *mPer*^{2Luc} transgenic mice [84]. The phase-shifting effects of ghrelin are present only when applied during the subjective day. This is in line with the notion that the SCN is more sensitive to the phase-shifting effects of non-photic cues during the day [85]. Similar phase advances are elicited in vivo by using GHRP-6, a synthetic ghrelin receptor agonist. When injected to mice in the middle of the light period, GHRP-6 elicited phase shifts in the locomotor activity of free-running animals [84]. The effects were present only under fasting conditions suggesting that feeding-induced signals may interfere with actions of ghrelin on the circadian clock.

Ghrelin signaling may play a role in the integration of photic and non-photic cues in the circadian system by attenuating the effects of light on the SCN. When a short, 10-min light pulse is applied early in the subjective dark, it elicits a phase

delay in the wheel-running activity of mice and stimulates c-fos expression in the SCN. Systemic injection of GHRP-6 before the light pulse attenuates the light-induced shifts in wheel running and the activation of c-fos in rat SCN [85]. It is possible that circulating ghrelin has a direct effect on SCN function since ghrelin is known to cross the blood–brain barrier [86, 87], ghrelin receptors are expressed in the SCN, and, as reviewed above, ghrelin has a direct effect on SCN activity in vitro. In addition, the SCN can be modulated by ghrelin through indirect mechanisms. One such mechanism involves the ARC. ARC is a main target for circulating ghrelin to stimulate feeding. The ARC may also play a role as an important interface between metabolic signals and the circadian system. There are reciprocal connections between the ARC and the SCN as evidenced by neuronal tracer [88] and in vivo extracellular electrophysiological studies [89]. It is proposed that ghrelin may serve as a peripheral metabolic signal in the function of the ARC–SCN unit. Systemic injection of GHRP-6 during the light phase induces increased fos expression in the ARC with the simultaneous suppression of fos activity in the SCN [88]. Another mechanism through which circulating ghrelin may affect circadian systems is related to the overall activating effects of ghrelin. Increased arousal itself interferes with the phase-shifting effects of light [90]. Ghrelin administration stimulates arousal, and causes increases in wakefulness leading to an overall behavioral activation [18, 21, 23]. The exact mechanisms through which increased arousal and the circadian system interact are unknown but likely involve pathways through which other non-photic effects modulate SCN activity such as connections from the raphe system and the intergeniculate nucleus.

Ghrelin and the Food-Entrainable Oscillator

Not only light but also regular, periodic feeding restricted to a few hours of the day is capable of entraining free-running behavioral rhythms and triggers the development of food-anticipatory responses such as increased behavioral activity, corticosterone secretion, and body temperature, 1–4 h before scheduled feeding time (reviewed in [82]). This food anticipatory activity (FAA) is driven by a biological clock, called the FEO, which is located outside the anatomical confines of the SCN. In the last four decades, a series of genetic and neural lesion studies, including experiments using Ppg KO and ghrelin receptor KO mice, have been performed to identify the exact location and key molecular components of the FEO. To interpret the findings from these studies correctly, one needs to keep two basic principles in mind. One, FAA is characterized by both the timing and the intensity of the premeal anticipatory responses. Timing, i.e., the precision in the regularity of the anticipatory responses, is a clock function. The intensity of a given response (e.g., number of wheel revolutions for wheel running, the amplitude of body temperature, or hormone responses) is affected by numerous clock-independent factors that influence the activity of the effector mechanisms involved. Two, the elimination of a signal that plays a significant role in the intrinsic clock machinery or is a key component

of its entraining input will interfere with timekeeping; thus it will prevent the manifestation of all response components of FAA. The attenuation or even the complete lack of one or more of these clock-driven effector activities cannot be interpreted as the lack of clock function as long as the reliable, precise timekeeping persists for at least one of the activities.

Despite the great efforts spent by multiple laboratories on identifying the location of the FEO, the site of the FEO remains elusive. Lesions of hypothalamic and extrahypothalamic neural structures all failed to abolish the timekeeping function of the FEO although the intensity of one or more of the food-anticipatory responses is often affected by a certain lesion (reviewed in [91]). Genetic deletion or postnatal elimination of feeding-related signals also failed to prevent FAA. Clock genes are expressed in various peripheral organs, including those of the gastrointestinal system, raising the possibility that the FEO may be located in the gastrointestinal system. Ghrelin emerged as an obvious candidate that may integrate feeding- and metabolism-related signals directed to the FEO or be part of the FEO itself. The secretion of ghrelin is locked to feeding activity; ghrelin plasma levels are elevated during fasting and suppressed after eating [78, 79, 92]. In scheduled feeding paradigms, plasma ghrelin levels increase in parallel with FAA [93].

The role of ghrelin in the FEO was investigated in three independent studies by using transgenic mice deficient in ghrelin signaling. In two studies using ghrelin receptor KO mice, food-anticipatory motor activity was measured either as wheel running activity or spontaneous locomotion, or both [94, 95]. In both experiments, clear entrainment to scheduled feeding develops in these KO mice indicating the integrity of the clock and its input signaling. In one experiment, the intensity of anticipatory wheel running did not differ between genotypes, but ghrelin receptor KO animals started running closer to the feeding time as compared to WT controls [95]. Timing and condensing increased activity to a shorter premeal anticipatory period could be considered a more efficient anticipation, an improved clock function. In contrast, in the experiment [94], the intensity of the anticipatory response, as measured by the number of wheel rotations or spontaneous activity counts, was attenuated [94]. This likely reflects a change in the activities of the effector mechanisms driven by the FEO, not an impaired clock function.

In a more comprehensive third study, Ppg KO mice were used and three parameters of FAA were measured simultaneously. Both normal and Ppg KO mice developed anticipatory increases in waking time, motor activity, and body temperature by day 6 of the scheduled-feeding paradigm. The intensity of these FAA responses, as measured by amounts of wakefulness and motor activity scores during the hour before the scheduled feeding and by the rate of premeal raise in body temperature, was not different between the two genotypes [59].

The usual word of caution applies to the interpretation of the findings in ghrelin receptor or Ppg KO animals. The findings that clear entrainment persists to scheduled feeding in both ghrelin receptor and Ppg KO mice indicates that the timekeeping function of FEO does not require intact ghrelin signaling. It is unlikely, therefore, that ghrelin plays key roles either as an input signal to the FEO or as an integrative component of the FEO machinery itself. Further, FEO-driven effector mechanisms

responsible for increased waking and spontaneous motor activity or body temperature responses do not require intact ghrelin signaling. Those effector mechanisms that are related to wheel running may involve ghrelin signaling. It is possible that while ghrelin is not part of FEO machinery or its input signaling, the secretion of ghrelin is driven by the FEO.

Ghrelin and Temperature Regulation

The relationship between thermoregulation and the regulation of metabolism has long been recognized. Adaptive metabolic heat production, non-shivering thermogenesis, is the major effector mechanism in cold adaptation and long-term cold defense. Adaptive thermogenesis is modulated by hormones and neuropeptides, most of which are also involved in the regulation of several other aspects of metabolism. The role of ghrelin in regulating feeding, adiposity, and metabolism in general is under intensive investigation (see Chap. 4 and 6 for details). In spite of these concerted efforts, a clear understanding of the effects of ghrelin on body temperature and its role in temperature regulation has not yet emerged. It is well established that ghrelin stimulates energy input, i.e., feeding, and the effects of ghrelin on the output side of the energy balance, i.e., metabolic heat production/energy expenditure, are surprisingly poorly understood.

Effects of Exogenous Ghrelin Administration on Energy Expenditure, Brown Adipose Tissue Function, and Body Temperature

Available data on the effects of exogenous ghrelin on thermoregulation and energy expenditure are limited and often contradictory. The studies focused on the acute or long-term effects of ghrelin on energy expenditure, brown adipose tissue (BAT) function, and body temperature. The acute effect of ghrelin on metabolic heat production appears to depend on the route of administration. Bolus icv injection of rat ghrelin into mice decreases energy expenditure [96] but systemic treatment has no effect [92]. Injection of an antibody that suppresses plasma levels of acylated ghrelin by 90%, but does not affect desacyl-ghrelin, increases energy expenditure in fasted mice but not after refeeding [97]. In another study, rats decrease energy expenditure in response to long-term, 14-day, icv infusion of ~10 nmol/kg/day human ghrelin [98]. In contrast, 10-day icv infusion of a lower dose (1.2 nmol/kg/day; [92]) or 6-day infusion of a much higher dose (2.5 nmol/day, ~70–80 nmol/day/kg; [99]) of rat ghrelin was ineffective.

The effects of icv injection of ghrelin on core body temperature are also somewhat ambivalent. Thus, orexigenic doses of ghrelin (0.1 and 1 μg) induce a transient 1–1.5 °C hypothermic response in rats [44]. In contrast, in another study, 1 and 5 μg

ghrelin elicited long-lasting fever-like responses which were significantly delayed and attenuated by a cyclooxygenase inhibitor [21]. In anesthetized rats, infusion of 1 nmol (3.3 μg) ghrelin had no effect on rectal temperature [100]. In mice, systemic injection of ghrelin deepens hypothermic bouts in fasted mice exposed to low ambient temperature [101].

It appears that ghrelin suppresses BAT activity, a major mechanism for nonshivering thermogenesis. The acute effects of ghrelin are due to the suppression of sympathetic efferents that innervate BAT. Infusion of ghrelin into the third ventricle suppresses the activity of the BAT sympathetic nerve and induces a rapid drop in BAT temperature in rats [100]. Acute icv, intra-PVN, intra-ARC, or iv injections of ghrelin suppressed norepinephrine release in BAT [102]. While systemic or icv single bolus injections of ghrelin do not affect uncoupling protein 1 (UCP-1) expression in BAT [102], the long-term effects of ghrelin on BAT function likely involve the downregulation of UCP-1 mRNA expression ([103] but see also [104]). The effects of ghrelin on UCP-1 expression require the intact sympathetic innervation of the BAT [99].

Transgenic Mice

In Ppg KO mice [105, 106] or ghrelin receptor KO mice [105–107] the energy expenditure is normal. Ppg and ghrelin receptor double-KO mice, however, show increased energy expenditure [106]. Age may be a significant factor in the role of ghrelin in regulating energy expenditure. Energy expenditure of Ppg KO and WT mice does not differ on standard chow diet or on high-fat diet when it is introduced at an older age [108]. When young, 6-week-old, KO mice are exposed to high-fat diet, they increase their energy expenditure compared to controls [109]. Also, 23-week-old Ppg KO mice have higher energy expenditure and lower respiratory quotients than WT controls; this difference disappears in older (54 week) animals [110].

At thermoneutral ambient temperature (30 °C for mice) there are no signs of gross thermoregulatory deficits in Ppg KO mice as they maintain normal body temperature comparable to that of WTs [59, 111]. Similarly, body temperatures of Ppg KO and ghrelin receptor KO mice do not differ from their respective WT controls under "standard housing" conditions [105]. Ppg KO mice respond to refeeding after a short-term fasting period with increased diet-induced thermogenesis [59]. Diet-induced thermogenesis is often attributed to increased BAT heat production [112]. Increased diet-induced thermogenesis in the absence of ghrelin signaling is consistent with the notion that ghrelin decreases metabolic heat production and BAT activity [96, 100].

At subthermoneutral ambient temperature (17 °C), Ppg KO mice show increased cold sensitivity manifested as significantly reduced body temperature and suppressed sleep compared to WTs. If WT mice are fasted at low ambient temperature, they develop transient hypothermic bouts of ~3–5 °C accompanied by increased

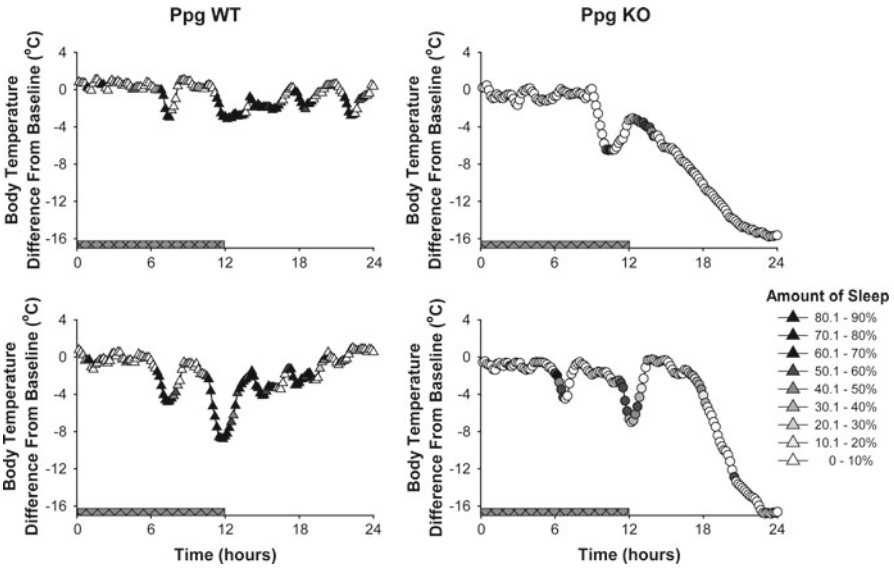

Fig. 8.2 Body temperature (*Y* axis) and the amount of sleep (darkness of symbols) in two individual preproghrelin wild-type (Ppg WT; *left panels*, *triangles*) and two Ppg knockout (KO; *right panels*, *circles*) mice fasted at 17 °C ambient temperature. Starting from hour 6, Ppg WT mice entered multiple hypothermic bouts during which body temperature dropped below normal by 2–10 °C. These periods of low body temperature were associated with increased sleep as indicated by the darker symbols. Body temperature in Ppg WT mice returned to normal by the end of the 24-h fasting. Ppg KO mice showed similar 2–8°C hypothermic bouts during the dark phase but these bouts were not accompanied by increased sleep as shown by the lighter color of the symbols. In the second half of the light period body temperature of Ppg KO mice started to drop precipitously and reached near-ambient temperature levels. When body temperature dropped below ~30 °C (i.e., 8–9 °C below baseline), vigilance states of mice were unidentifiable by using standard electroencephalographic criteria. *Horizontal grey bars*: Dark period

NREMS; by the end of the 24-h fasting period in cold, body temperature of WT mice returns to normal (Fig. 8.2). In Ppg KO mice, however, fasting in cold exacerbates their thermoregulatory deficiency [111]. During the first 16 h of fasting, KO animals show similar hypothermic bouts as WT mice but sleep increases do not accompany these drops in core temperature. Subsequently, body temperature of KOs drops precipitously and it reaches near-ambient temperature. By the end of the 24-h fasting, EEG-defined sleep disappears and mice had to be passively rewarmed to prevent death. Interestingly, ghrelin receptor KO mice show no such sensitivity to combined cold and fasting challenge. This suggests that the thermoregulatory and sleep deficits in Ppg KO mice are not due to the lack of ghrelin signaling. Replacement of obestatin, the other major Ppg gene product, by using osmotic minipumps partially rescued the phenotype; it delayed the onset of the hypothermic response by 6 h in Ppg KO mice exposed to fasting in cold [111]. This suggests that lack of obestatin in Ppg KO mice may, at least in part, be responsible for the observed thermoregulatory deficit.

Summary

Ghrelin is a member of the group of neuropeptides/hormones that play a role in the regulation of vigilance. Animal studies show that ghrelin signaling is a component of the arousal system. While the exact role of the circulating ghrelin pool in the regulation of vigilance is not yet clear, central ghrelinergic mechanisms—as part of the hypothalamic ghrelin–orexin–NPY circuit—are posited to play a role in promoting wakefulness and feeding (Fig. 8.3). The functional significance of the circuit is to integrate metabolic and circadian signals with vigilance status. Data from human studies are less clear and often contradictory. Due to the cost and the inherent complexity of human sleep experiments, crucial studies, such as establishing dose–response relationships for ghrelin, are lacking. Furthermore, human studies only investigate the function of the circulating ghrelin pool and give only limited information about central ghrelinergic mechanisms. Ghrelin modulates the activity of the main circadian clock, the SCN. It is unlikely, however, that it is a crucial component of or a key input signal to the FEO.

Ppg KO and ghrelin receptor KO models are inconclusive due to negative or contradictory results. The arousal system is highly redundant; its core component is formed by multiple diffuse ascending projections arising from multiple forebrain, midbrain, and brain stem sites. A single key, permissive signaling component in the sleep–arousal system has not been identified. Eliminating individual signaling components, such as ghrelin, orexin, or histamine, gives rise to fragmented sleep phenotypes under normal conditions. When the arousal system is stimulated by exposure to novel environments or fasting, ghrelin receptor KO mice show deficient arousal responses. This is consistent with the hypothesis that ghrelin signaling plays a role, at least under certain conditions, in the function of the arousal system. Ppg KO animals do not show signs of major thermoregulatory

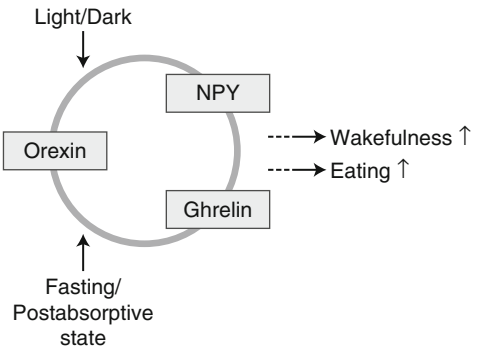

Fig. 8.3 The ghrelin–orexin–neuropeptide Y (NPY) circuit in the hypothalamus integrates circadian (light–dark) and metabolic signals during fasting/postabsorptive states. The activation of the circuit is manifested in the dark-onset syndrome in nocturnal rodents. Characteristics of the dark-onset syndrome are increased wakefulness and feeding activity

deficits under normal conditions. Products of the Ppg gene, particularly obestatin, are likely to be involved, however, in maintaining normal body temperature and metabolism under conditions where increased metabolic heat production is required. There are still unanswered questions about the significance of ghrelin signaling in regulating energy expenditure and its role in central thermoregulatory circuits. Our understanding of the physiological role of ghrelin expanded remarkably in the 14 years since the discovery of the hormone. Yet, full comprehension of its role in the regulation of vigilance, circadian clock, and body temperature remains incomplete.

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Chapter 9
Ghrelin Regulation of Learning, Memory, and Neurodegeneration

Tamas L. Horvath

Abstract Late-onset chronic diseases, such as dementias, Alzheimer's and Parkinson's disease, diabetes, cardiovascular disorders, and tissue malignancies, are the leading causes of morbidity and mortality in the USA, creating the greatest emotional and financial burden on the individual and society. As the size of the aging population continues to grow, late-onset chronic diseases are predicted to further dominate the attention of biomedicine and society at large. Guided by the notion that late-onset chronic diseases are the consequence of prolonged overworking of various tissues that have genetic and epigenetic vulnerabilities, it is reasonable to suggest that it is the cellular energy metabolism of the different tissues that determines their health and longevity and consequently those of the entire organism. Ghrelin is a gut-derived hormone that affects brain functions at the time of low nutrient availability from the environment. Because such conditions, including calorie restriction, have been shown to suppress chronic disease development and prolong longevity, ghrelin may act to enable the propagation of these central and peripheral mechanisms. Here I review the conceptual and experimental aspects of ghrelin's action on integrative physiology in the promotion of brain health and life span.

T.L. Horvath, D.V.M., Ph.D. (✉)
Yale Program in Integrative Cell Signaling and Neurobiology of Metabolism,
Section of Comparative Medicine, Yale University School of Medicine,
300 George Street, Suite 203, New Haven, CT 06511, USA
e-mail: tamas.horvath@yale.edu

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Introduction

The presence of a central nervous system (CNS) is not necessary in the support of primitive forms of life. Indeed, a CNS is not found in simple biological entities nor does it exist in some complex living organisms such as plants. However, for the successful maintenance and propagation of multicellular animal species, a coordinator of cellular and tissue activities is mandatory. In developed animal species as well as in humans, the CNS serves this purpose. It enables the coordinated activities of internal organs (autonomic and endocrine functions) with those of the musculoskeletal system (behavior) in support of survival in the environment and for the successful maintenance of the gamete through reproduction. The oxidation of cellular substrates determines life. Thus, by applying evolutionary principles, it is reasonable to argue that the more sophisticated the CNS the higher the likelihood for survival due to more effective promotion of cellular substrate oxidation. In the presence of a relatively steady atmospheric environment, daily survival hinges on an organism's ability to acquire fuel from the environment and to escape predatory threats. Hence, it is appropriate to assume that the activity of the CNS is to respond to these environmental challenges.

Hunger as a Driver of Mnemonic Functions

Historical information from art, literature, and science argues that *appetite* is a driver of invention, creativity, and civilization, in general. From the migration of our ancestors north out of Africa through the exploration of outer space, the history of the evolution of human society has been characterized by innovations associated with seeking and securing resources in support of survival. Besides these common sense truisms, there is an incredible amount of irrefutable experimental evidence in the scientific literature over the past 100 years in support of the relevance of hunger as a primary driver of higher brain functions.

Experimental neuroscience from the time of Pavlov to the present day has utilized approaches of positive re-enforcement that hinge upon hunger or appetite as the motivational force to learn new tasks, which can then be manipulated by various means and subsequently analyzed to determine the contribution of specific pathways or signaling modalities. In a vast array of studies employing model systems of invertebrates, as well as of rodent and nonhuman primates, food deprivation has been used as a means to better understand cognitive functions, learning and memory [1]. While there are many other methods for the study of mnemonic functions, the profound role that hunger plays in promoting complex brain functions is evident from the thousands of studies that have been undertaken over the past ten decades [1]. Although this effect of hunger has usually been taken for granted in these methodologies, recently the humoral, neuronal, and molecular contributors of this phenomenon have emerged and suggest a fundamental organizational role of the hypothalamus and peripheral tissues in this process.

Empirical Evidence for the Fundamental Role of Hypothalamic NPY/AgRP Neurons in Hunger

Sixty years ago, studies revealed that localized hypothalamic lesions can lead to the cessation of feeding and subsequent death [2–6]. This lethal intervention in hypothalamic structure is different from that following specific lesions in the hindbrain that cause immediate death due to the stoppage of breathing and/or heart function, because death caused by hypothalamic lesions takes several days. In the past couple of decades, two key peptidergic systems were discovered in the lateral hypothalamus where lesions cause aphagia and death: the hypocretin/orexin neurons [7, 8] and melanin-concentrating hormone (MCH)-producing cells [9]. Both of these neuronal systems, when activated, promote feeding [8, 10]. Despite this, selective acute elimination of either of these neuronal populations failed to replicate the effect of lateral hypothalamic lesions [11, 12]. Strikingly, however, selective ablation of a small subset of inhibitory neurons in the hypothalamic arcuate nucleus successfully recapitulated the effect of the classic lesion studies [13, 14]. These neurons are those that coproduce neuropeptide Y (NPY), Agouti-related protein (AgRP) [15], and gamma amino butyric acid (GABA) [16].

A fundamental role for the hypothalamic arcuate nucleus NPY/AgRP neurons in the regulation of feeding had been suspected for more than 25 years. First, in 1984, NPY was found to be the most potent inducer of appetite and feeding ever described when applied to the cerebral ventricle [17]. Second, NPY neurons were anatomically [18] and functionally [19] tied to the hypothalamic melanocortin-producing neurons which play a fundamental role in satiety [20].

By the beginning of this millennium, a consensus emerged that the NPY/AgRP/GABA neurons serve as critical suppressors of the melanocortin system [20], a tonic inhibitory relationship supported by both hard wiring [18] as well as pre- and postsynaptic actions [19]. This tonic inhibition is the underlying cause for appetite and feeding stimulation being set as the default signaling of the brain, an obvious necessity from the perspective of survival. Because of this, it was surprising that the removal of neither NPY nor AgRP by means of knockout technology did not lead to major alterations in the metabolic phenotype [21, 22]. However, when the technology to selectively eliminate these neurons in adult animals became available, a striking observation was made: an acute, selective elimination of AgRP neurons in adult mice lead to the cessation of feeding [13, 14] and, ultimately, death [14]. This effect is not an artifact of acute neuronal degeneration within the arcuate nucleus as asserted by studies that showed that when the neighboring pro-opiomelanocortin (POMC) neurons, the activation of which is associated with satiety, are eliminated in the same manner, animals are fine and actually eat more [13].

The moribund state of AgRP neuronal ablated animals is characterized by an overall disinterest in the environment and the lack of desire for food for days preceding death [14, 23, 24]. This lack of overall interest in the environment despite imminent death is a hallmark of end-of-life cachexia associated with advanced age and/or certain types of cancers. The lethal effect of this intervention (AgRP

neuronal ablation) can be blocked by site-selective administration of agonists of the inhibitory neurotransmitter, GABA [24], an amino acid transmitter present in arcuate nucleus NPY/AgRP neurons [16]. The observation that the acute elimination of AgRP neurons (only a few thousand neurons are present in the hypothalamus) blocked all aspects of animal behavior that enables survival (with the exception of breeding) raises the remarkable possibility that these neurons are master regulators of higher brain functions that promote the successful interaction with the environment in support of survival.

The hypothalamic AgRP system is a predominant inhibitory [16, 19] neuronal system providing input to various hypothalamic nuclei as well as to brainstem regions [25]. The outputs are primarily descending projections to phylogenetically older areas with the notable exception of a strong projection to the paraventricular thalamic nucleus and some sparse efferents to limbic regions such as the bed nucleus/lateral septal area and the medial amygdala. Regarding its significant "life"-supporting action, the parabrachial nucleus (PBN) in the midbrain–hindbrain junction was identified as the key relay of AgRP neuronal signaling [24, 26]. The PBN is suggested to mediate signals from the gut via the nucleus solitary tract to higher brain regions. Presently, however, the fine blueprint is unclear with regard to the neuronal signaling cascade that is triggered by the NPY/AgRP neurons that enables the motivational and cognitive adjustments by higher brain regions to support continuous interest in the environment to seek food and to survive. Beyond the neuronal route of action of these arcuate nucleus cells on higher brain functions, however, there is a clear and pronounced effect of negative energy balance evoked by the NPY/AgRP neurons on various autonomic and endocrine processes. Intracellular carbohydrate and fat metabolism in the neuronal substrate of the melanocortin system is an important regulator of the autonomic and endocrine outputs of the hypothalamus [27–34].

Ghrelin, a Humoral Link Between Hunger and Higher Brain Functions

Signals from the periphery initiate a brain response to declining energy availability. These signals arise from various peripheral tissues in the form of hormones and nutrients. Of the various hormones, only one to date has been shown to trigger feeding behavior, and that is the gut-derived acylated polypeptide, ghrelin [35, 36]. Ghrelin not only triggers feeding behavior by activation of the arcuate nucleus NPY/AgRP neurons [37], but also exerts action in extra-hypothalamic sites as well. Specifically, ghrelin was shown to affect excitatory synaptic transmission within the hippocampal formation [38] and to alter the activity of midbrain dopamine neurons [38, 39].

Ghrelin was originally discovered as a regulator of growth hormone release [35], and subsequently was identified as an appetite-stimulating, adipogenic hormone [36]. Ghrelin is secreted from the stomach, when the stomach is empty. Thus, a

major emphasis of ghrelin research has been focused on its possible roles in meal initiation and energy homeostasis, in general.

Numerous studies revealed that the effect of ghrelin to promote feeding is mediated by the arcuate nucleus AgRP neurons [37, 40]. In slice preparations, AgRP neurons were activated by ghrelin directly, while the anorexigenic POMC neurons were inhibited indirectly by ghrelin [37, 40]. Intriguingly, besides the acute electrophysiological effects of ghrelin on these neurons, peripheral ghrelin administration also rapidly reorganized the synaptic inputs of POMC neurons [41]. The resultant synaptic rewiring of the POMC neurons further promoted the suppression of these arcuate cells, which is consistent with the observation of an overall orexigenic influence of ghrelin.

However, ghrelin that originates in the periphery also controls higher brain functions and may represent a molecular link between learning capabilities and energy metabolism. For example, circulating ghrelin enters the hippocampal formation and midbrain where it binds to neurons [42]. Ghrelin receptors are expressed in neurons of these extra-hypothalamic sites, and ghrelin promotes the formation of synapses in both of these areas. Ghrelin-regulated synapse formation and long-term potentiation of synapses in the hippocampus have a positive correlation with spatial memory and learning [42]. Beyond the alteration of these mnemonic functions, hippocampal administration of ghrelin also promoted feeding [43].

The interference of ghrelin signaling in the midbrain also suppressed feeding triggered by peripheral ghrelin administration, which argues for a role of the midbrain reward circuit in feeding regulation. However, the dopamine system of this area also projects to the prefrontal cortex suggesting a direct role of ghrelin in motivated behavior resulting from cortical modulation and in working memory as well.

The ventral tegmental area is in the immediate vicinity of the substantia nigra, where dopamine neurons are critically involved in the regulation of motor functions. The loss of this dopamine system is the underlying cause of Parkinson's disease, and ghrelin was recently found to have a robust action on these cells in which it promotes and protects the activity of the dorsostriatal dopamine system [39]. Elegant experiments have also revealed that besides the well-known effect of this dorsostriatal dopamine system in the regulation of movement, dopamine signaling in this pathway has direct relevance to feeding behavior as well [44–46].

The preceding examples of ghrelin action highlight the emerging view that humoral signals associated with energy metabolism have profound and direct effects on brain areas that have not classically been associated with behavioral, endocrine, and autonomic regulation of peripheral tissue functions. Although this review focuses on signals of hunger, the adipose hormone, leptin, that promotes satiety has also been shown to impact these structures and associated brain functions, such as motivated behavior, learning, and memory [47].

Functional imaging studies of the human brain confirm that these effects of ghrelin target the same brain sites that were unmasked in experimental animal models [48–50]. All and all, these seemingly novel and interesting findings simply emphasize the relevance of the assertion that the function of all aspects of the CNS is under the control of the metabolic needs of the body and that peripheral tissues exert their

requirements by shifting the activity of various brain regions, in part, by humoral signals. Intriguingly, however, the hypothalamic AgRP neurons appear to play a critical organizational role in this effect of peripheral tissue regulation of brain functions upstream of both the periphery and higher brain regions: their selective inactivation in the adult animal leads to a cessation of ghrelin's effect on feeding, eliminates behaviors associated with metabolic needs and survival, and results in death [14, 23, 24]. It is likely that the underlying cause of this effect is mediated by both hormonal dys-regulation (impaired peripheral hormone responses to negative energy balance) and improper adjustment of fuel reallocation by peripheral tissues to support brain functions.

Fatty Acid Metabolism in the Brain Is Key to Ghrelin's Action

A shift in metabolic status is paralleled by a shift in the availability of nutrients in the circulation. Specifically, a state of hunger is characterized by increased circulating free fatty acid levels (released mainly by the white adipose tissue) and decreased circulating carbohydrate levels (mainly produced by the liver). Just how these changes in fuel availability affect brain functions is not clear at present and is the intense focus of many current investigations.

There is a long-held view that neurons utilize carbohydrates as fuel for firing action potentials, mainly glucose metabolites provided, in part, by glial cells. Under extended negative energy balance, ketones are considered as an alternative fuel for brain cells. On the other hand, several textbooks explicitly state that under no circumstance do neurons utilize fatty acids as fuel. These assertions and dogmas have recently been challenged, and data is emerging to indicate that fuel availability, utilization, and efficiency to promote firing of neurons may be more complex than originally thought and are under the control of the peripheral energy status and tissue metabolism.

While the majority of neurons in the brain utilize carbohydrates, glucose, and lactate, as fuel for the generation of action potentials in a wide range of glucose availability, subset of neurons specifically in the hindbrain and hypothalamus only fire at high glucose concentrations (glucose-activated neurons) [51, 52]. On the other hand, other sets of cells in the hindbrain and hypothalamus fire only at low glucose concentrations when circulating long-chain fatty acids are elevated due to mobilization of fat stores during hunger [51, 52]. The activation of hypothalamic POMC neurons coincides with feeding-associated rise in circulating and brain glucose concentrations and declining active ghrelin levels. The firing of these cells results in cessation of feeding and promotion of insulin secretion and subsequent glucose utilization by all tissues [30]. The period of satiety is accompanied by adjustments in all tissue functions to enable the most efficient utilization and storage of acquired nutrients. At this time, locomotor activity is diminishing and all brain functions are aligned to support the synchronization of peripheral tissues via autonomic and neuroendocrine pathways for the most efficient processing of nutrients. For example, this is the time for least efficient learning, while the storage of memory is being strengthened.

Seek to acquire is characteristic of negative energy balance/hunger, when circulating and brain glucose levels are lowering, circulating fatty acid levels are rising, and active ghrelin is increasing in the circulation. This shift in fuel availability in the brain is the time when ghrelin-driven AgRP neurons are firing at the highest rate [40, 53, 54] promoting behavioral, autonomic, and endocrine correlates of hunger, all of which are necessary for survival. Fat metabolism in the hypothalamus is a critical contributor to the shift of neuronal activity in support of hunger as indicated [28–34, 55]. We have shown that the machinery promoting intracellular fatty acid metabolism triggered by ghrelin is present in the AgRP neurons [40]. That it is a likely mediator of ghrelin's action on brain functions may be asserted from the findings that this same intracellular pathway is also present in other parts of the brain. For example, substantia nigra dopamine neurons were shown to operate with the same machinery during the time of negative energy balance as described in AgRP neurons [39], emphasizing the relevance of fatty acid utilization as fuel in neurons that promote locomotion during hunger. Locomotion/exercise and hunger all promote processes in learning. Both the humoral signal of hunger, ghrelin [42], as well as the aforementioned fatty acid utilizing pathway [53] have been associated with the hippocampal formation, primary site of the machinery enabling spatial learning. When a critical component in neuronal fatty acid utilization was eliminated, voluntary exercise failed to promote new synapse formation in the dentate gyrus [53], a critical process in learning and memory formation [54].

Based on the above, it is reasonable to assert that a shift in fatty acid and glucose metabolism driven by ghrelin is a key component of ghrelin-induced adaptation of brain functions and promotion of tissue integrity.

Ghrelin as a Driver of Tissue Integrity

Whether the fuel is carbohydrate or fatty acids for neuronal firing, the by-product of substrate oxidation are free radicals. Recent data argue that ROS generation is not simply a by-product of substrate oxidation, but rather a critical role player in regulating neuronal responses in a substrate-dependent manner [40]. For example, when circulating active ghrelin levels are high during negative energy balance, ROS levels are not increased in the brain despite increased firing and substrate utilization by some neurons [40]. In contrast, when active ghrelin levels are low during positive energy balance and glucose-utilizing neurons are firing at high levels, ROS levels increase in these cells [40].

Late-onset chronic diseases, including dementias, Alzheimer's and Parkinson's disease, diabetes, cardiovascular disorders, and tissue malignancies, are the leading causes of morbidity and mortality creating the greatest emotional and financial burden on the individual and on society as a whole. As the size of the aging population continues to expand, late-onset chronic diseases are predicted to further dominate the attention of biomedicine and society at large.

Guided by the notion that late-onset chronic diseases are the consequence of the prolonged overworking of various tissues that have genetic and epigenetic

vulnerabilities, one might assert that it is the cellular energy metabolism of the different tissues and their synchronous activity that determines health and longevity. In support of this idea is the positive effect seen of calorie restriction on healthy life span. Numerous studies in various models, including worm, fruit fly, mice, rats, and nonhuman primates, showed conclusively that moderate calorie restriction prolongs life, both mean and maximal life span. To date, this is the only physiological intervention that has consistent and predictable effects to prolong life and health in all species studied. In humans, prospective experiments are under way, and preliminary data show that similar shifts in metabolic profiles and overall tissue health are also associated with calorie restriction in men and women. These robust effects of calorie restriction, a form of subtle, chronic negative energy balance, lend support to the argument that late-onset disorders are the consequence of sustained high levels of substrate oxidation by cells of various tissues. In the preceding paragraphs, evidence was provided that the key cellular entity involved in promoting central and peripheral attributes of negative energy balance (including that occurring during calorie restriction) is promoted by active ghrelin and neuronal fatty acid utilization. Thus, it is logical to argue that ghrelin plays an important role in the determination of healthy tissue function and longevity.

Summary

Ghrelin has the capacity, through direct neuronal and indirect peripheral actions, to coordinate mechanisms that are implicated in higher brain functions and in the etiologies of chronic disorders of diverse tissues, such as the brain, immune system, liver, pancreas, muscle, lung, and heart. The breadth of knowledge that has been acquired regarding the site and intracellular mode of action of ghrelin will allow us to apply these tenets to other physiological and pathological processes to reveal novel approaches in the treatment of various tissue dysfunctions, such as immune deficiencies and dementias.

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Chapter 10
Ghrelin Antagonism: A Potential Therapeutic Target for Addictive Behaviour Disorders

Elisabet Jerlhag, Emil Egecioglu, Jörgen Engel, and Suzanne L. Dickson

Abstract Here we review the emerging evidence for a role of the central ghrelin signalling system in reward from food (i.e. a natural reward) as well as from alcohol and other drugs of abuse (i.e. artificial rewards). Ghrelin levels are high preprandially consistent with a role in hunger and meal initiation. The hypothalamic and brainstem circuits involved in energy balance are clearly important targets for ghrelin. However, ghrelin also activates a key reward circuit, the cholinergic–dopaminergic reward link, that is involved in motivated reward-driven behaviour. This reward link comprises a dopamine projection from the ventral tegmental area (VTA) to the nucleus accumbens together with a cholinergic input, arising primarily from the laterodorsal tegmental area. Direct injection of ghrelin into the brain ventricles or into the VTA increases the consumption of rewarding foods as well as alcohol in mice and rats. Conversely, ghrelin receptor (GHS-R1A) antagonists suppress reward from chemical drugs and also from rewarding foods, thereby decreasing their consumption. Variations in the GHS-R1A and pro-ghrelin genes have been associated with high alcohol consumption, smoking and increased body mass index in alcohol-dependent individuals as well as with bulimia nervosa and obesity. Thus, the central ghrelin signalling system is strongly implicated in reward from food as well as chemical drugs, thereby providing a potential therapeutic target for addictive behaviour disorders, including those associated with compulsive overeating, obesity as well as substance use disorder.

E. Jerlhag, Ph.D. • J. Engel, Ph.D., M.D.
Department of Pharmacology, University of Gothenburg, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, Gothenburg, Sweden

E. Egecioglu, Ph.D., M.Sc.
Department of Physiology, University of Gothenburg, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, Gothenburg, Sweden

S.L. Dickson, B.Sc., Ph.D. (✉)
The Sahlgrenska Academy at the University of Gothenburg, Institute of Neuroscience and Physiology, Medicinaregatan 11, Gothenburg 436 44, Sweden
e-mail: suzanne.dickson@gu.se

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Introduction: The GHS-R1A as a Therapeutic Target: A Historic Perspective

The ghrelin receptor, GHS-R1A, has emerged as a potential therapeutic target for a number of diverse disease areas. In the 1990s, there was a general expectation that GHS-R1A agonists, by virtue of their stimulatory effects on the somatotrophic axis, would become a therapy to improve growth, have beneficial anabolic effects on bone and muscle and, by increasing lipolysis, reduce body fat. Our research programme investigating potential fat-reducing effects of chronic GHS-R1A agonist treatment came to an abrupt end when our mice paradoxically became obese [1]. Indeed, while attempting to provide a mechanism to explain these adipogenic, growth hormone-independent effects of GHS-R1A agonists, Matthias Tschöp and colleagues reported that the newly discovered endogenous ligand for GHS-R1A, ghrelin, increases fat mass by a mechanism that includes decreased fat utilization [2]. This seminal discovery marked a new era in therapeutic development of GHS-R1A, with new focus on the identification of potent antagonists or inverse agonists for this receptor that would provide a blockbuster therapy for the treatment of obesity. This potential therapeutic use for such compounds gained further impetus by the discovery that ghrelin may operate as a circulating hunger hormone with a role in meal initiation [3]. Recently, however, we proposed an entirely novel disease area for the use of GHS-R1A antagonists, namely, substance abuse, including alcohol use disorder [4].

Here we review the evidence base that ghrelin targets key mesolimbic reward circuits and that its action at this site is important for reward from alcohol and, indeed, from other chemical drug rewards. Moreover, these findings are discussed together with the emerging clinical evidence that the central ghrelin signalling system provides an interesting therapeutic target for addictive behaviour disorders, as reviewed elsewhere [5].

Identification of the Midbrain Dopamine System as a Target for Ghrelin: Relevance for Addictive Behaviour Disorders

When Roy Smith and colleagues first described the CNS distribution of GHS-R1A in human and rat brain [6, 7], it was clear that it was expressed in many regions for which we could not immediately provide an obvious functional link. GHS-R1A expression in the hypothalamus (especially the arcuate nucleus) readily explained the role of GHS-R1A for the growth hormone-releasing effects and later, found relevance for effects on fat accumulation and energy balance. However it is only recently we have begun to understand the importance of ghrelin signalling in areas such as the hippocampus, where it has been linked to memory formation [8], and the mesolimbic and tegmental areas that are intimately associated with reward.

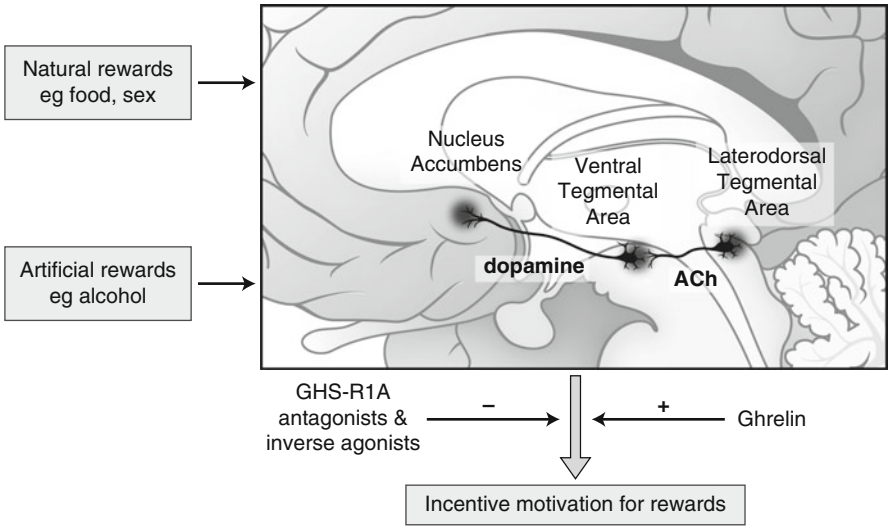

Fig. 10.1 The cholinergic–dopaminergic reward link, a target for ghrelin and ghrelin receptor (GHS-R1A antagonists), increases the incentive motivational value of natural and artificial rewards. It comprises a dopamine projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc) that is innervated by a nicotinic cholinergic input from the laterodorsal tegmental area (LDTg)

Ghrelin Activates the Mesoaccumbal Dopamine Pathway

In 2006, we provided evidence that ghrelin targets the mesolimbic dopamine pathway. This dopamine pathway originates in the ventral tegmental area (VTA) and sends projections to the nucleus accumbens (NAcc) (Fig. 10.1). Accumbal dopamine release is widely believed to mediate the rewarding properties of incentives, natural as well as artificial [9–11]. Thus, accumbal dopamine overflow is a common response to various reinforcers. Moreover, this dopamine system is intimately involved in incentive-motivated behaviour ("wanting") for reward reinforcers such as food [12]. Given that GHS-R1A are expressed in the VTA [7, 13] we hypothesized that ghrelin may activate the reward system. In a series of experiments we found that administration of ghrelin into the brain ventricles [14] or, indeed, into the VTA [15] increases locomotor activity as well as accumbal dopamine in mice (Fig. 10.2), indicating that ghrelin activates the mesoaccumbal dopamine system. Our initial findings were substantiated and extended by the group of Tamas Horvath, who provided neuroanatomical and electrophysiological evidence that these midbrain dopamine neurones are a target for ghrelin [16]. They found that as many as 60% of the VTA dopamine neurones express GHS-R1A, although this receptor was also expressed in other cells in this region, including a sub-population of the GABAergic cells. In their electrophysiological studies, cells were recorded in a slice preparation using whole cell patch clamp techniques; cells fulfilling criteria for identification as dopaminergic cells were excited by bath application of ghrelin.

Ghrelin, a new instrument in the brain reward orchestra?

	Locomotor activity	Accumbal dopamine release	Alcohol intake	Alcohol-induced locomotor activity	Alcohol-induced accumbal dopamine	Alcohol-induced CPP
Ghrelin (icv, intra-VTA, intra-LDTg)	↑	↑	↑			
GHS-R1A knockout			↓	↓	↓	↓
GHS-R1A antagonists (icv/ip)			↓	↓	↓	↓

Fig. 10.2 Summary of our recent data outlining the effects of ghrelin administration (into the brain ventricles or directly into the VTA or LDTg) on mice, as well as pharmacological (GHS-R1A antagonist) or genetic (GHS-R1A knockout) suppression of the central ghrelin signalling system on reward parameters (accumbal dopamine release and locomotor stimulation) and on alcohol's rewarding effects

Given the dynamic changes in ghrelin release in relation to mealtimes in human volunteers [3], together with its ability to induce hunger [17] and to increase food intake in rodents [18], it is conceivable that ghrelin may have, at least in situations without access to food, incentive value for goal-directed behaviours such as meal initiation and/or food searching. Consistent with this, accumbal dopamine release (which ghrelin activates) appears to be pivotal for determining the incentive motivational value of food [12]. Identification of the mesoaccumbal dopamine projection as an important brain target for ghrelin strongly implicates the central ghrelin signalling system in the reward mechanism. It also indicates that GHS-R1A may be a relevant clinical and therapeutic target for addictive behaviour disorders in which the dopamine system has been implicated, such as alcohol dependence, other substance use disorders and even certain eating disorders [19, 20].

Does Peripheral Ghrelin Act Directly in the VTA for Its Dopamine-Releasing Effects?

Ghrelin is mainly produced in and secreted from the stomach [21], raising the possibility that peripherally produced/administered ghrelin can activate the mesolimbic dopamine system. We found that peripherally (i.p.) administered ghrelin induces locomotor stimulation and accumbal dopamine release and conditions a place preference in mice [22]. Indeed, all of these parameters were

abolished/suppressed by intra-VTA administration of a ghrelin receptor antagonist, pinpointing the VTA as a primary site for eliciting these effects (Fig. 10.2) [23]. Supportively, systemic ghrelin increases accumbal dopamine turnover ex vivo and releases dopamine in the NAcc shell [24]. Collectively these findings suggest that peripheral ghrelin accesses the VTA where its dopamine-releasing effects are dependent on GHS-R1A signalling. It cannot be ruled out that centrally (hypothalamic) produced ghrelin [25] may also regulate the VTA dopamine system, although little is known to date about the wiring of these ghrelin-producing cells and whether they project to ghrelin-responsive reward targets such as the VTA and NAcc.

Additional Indirect Targets for Ghrelin's Dopamine-Releasing Effects

In addition to having direct effects on the mesoaccumbal dopamine system it now seems clear that ghrelin also has indirect effects. Consistent with this, within the VTA GHS-R1A is also present on pre-synaptic afferents [16], the identity of which appears to include both glutamatergic and cholinergic inputs [23]. An important part of the reward systems, for both natural as well as artificial reinforcement, is the so-called cholinergic–dopaminergic reward link (Fig. 10.1); for review see [26, 27]. This link encompasses the aforementioned mesoaccumbal projection together with a cholinergic projection from the laterodorsal tegmental area (LDTg) to the VTA [28, 29]. Natural rewards and dependence-producing drugs, in addition to increasing accumbal dopamine release, simultaneously enhance VTA acetylcholine (ACh) levels [27, 30–32]; this implicates the cholinergic–dopaminergic reward link in the hedonic and reinforcing aspects of natural rewards, that include food, as well as of addictive drugs, such as alcohol. GHS-R1A is expressed in the LDTg [7] and we found that direct administration of ghrelin into the LDTg of mice also increases locomotor activity and associated accumbal dopamine release (Fig. 10.2) [15]. The effects of ghrelin on dopamine release and locomotor activity, whether administered into the brain ventricles, VTA, or LDTg, could be blocked by peripheral administration of a non-selective nicotinic cholinergic antagonist, mecamylamine [14, 15]. Taken together with the findings that the LDTg provides a prominent source of cholinergic afferents to the VTA [27] and that the LDTg cholinergic cells express GHS-R1A [33] it seems likely that mecamylamine interrupts afferent cholinergic signals originating primarily in the LDTg. In studies using selective pharmacological blockers of nicotinic acetylcholine receptor signalling we were able to identify which particular nicotinic cholinergic receptor subunit compositions are important for ghrelin signalling. Our studies implicated the $\alpha_3\beta_2$*, α_6* and β_3* subunit compositions [34], a finding of particular interest as these receptor subtypes also have a key role in alcohol reward [26]. Supportively, an association between the α_6* *(CHRNA6)* gene and heavy alcohol use has been found [35]. Collectively these studies demonstrate that there are neurochemical analogies between alcohol and

ghrelin and that there is neurochemical overlap in the reward circuits through which ghrelin and alcohol operate, highlighting, in particular, the cholinergic–dopaminergic reward link as a common denominator.

Future therapeutic targets for addictive behaviour disorders that interrupt ghrelin signalling may also include agents, such as nicotinic cholinergic blockers, that interfere with the circuits through which ghrelin operates. Of the many potential additional afferent signalling systems that may be important for ghrelin-mediated effects, we highlight especially glutamatergic and orexin A pathways. We found that ghrelin's ability to activate the mesolimbic dopamine system was suppressed by pharmacological blockade of glutamatergic NMDA receptors but not by blockade of orexin A receptors [23]. Consistent with the effects on glutamate signalling, ghrelin's effects on the electrical activity of dopaminergic neurones in the VTA appear to be dependent upon excitatory glutamatergic input [16]. It has also been demonstrated that blockade of NMDA receptors in the VTA reduces food-induced accumbal dopamine release [36]. By contrast, the orexin receptor A antagonist did not impact upon ghrelin's dopamine-releasing effects as central orexin signalling has been shown to be important for ghrelin-induced food intake and for appetitive behaviour for high-fat feeding [37, 38], suggesting that food-seeking versus food intake/appetite are regulated, at least in part, via different mechanisms.

Central Ghrelin Signalling Is Required for Alcohol Reward

Alcohol dependence is a chronic disorder and is today recognized as a disease. Alcoholism causes considerable suffering to the individual as well as to their families and society [39]. Patient-related problems include decreased health status, malnutrition, liver damage and cardiovascular problems. Characteristics of the disease include compulsive drug-seeking behaviour and a loss of control. Attempts have been made to classify this heterogeneous disease into different subtypes, implying differential neurochemical, genetic and psychological causes as well as differential opportunities for treatment strategies.

For many years the Engel group has explored various instruments in the brain reward orchestra for which alcohol is a guest conductor (Fig. 10.3), culminating in the recent suggestion that the central ghrelin signalling system, involving GHS-R1A, may operate as a new modulator/amplifier of this system. Consistent with the hypothesis that ghrelin may increase the incentive motivational value of alcohol, we found that central ghrelin signalling is required for alcohol reward (Fig. 10.2) [4]. First, we demonstrated that ghrelin increased alcohol intake in a free choice (alcohol/water) limited access paradigm in mice, when administered into the brain ventricles or into the VTA or LDTg. By contrast, two different GHS-R1A antagonists, administered centrally or peripherally, reduced alcohol intake in this model. We also found that pharmacological or genetic suppression of GHS-R1A completely blocked the stimulatory effects of alcohol on locomotor stimulation and accumbal dopamine release. Finally, we found that a GHS-R1A antagonist suppressed the ability of

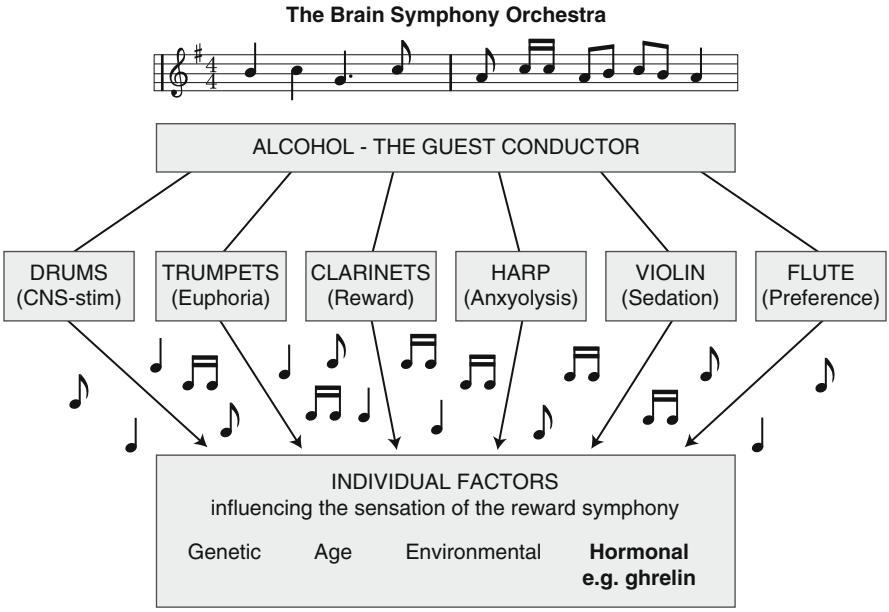

Fig. 10.3 The brain symphony reward orchestra for which alcohol is a guest conductor, first described by Jörgen Engel in 1992 [87], is a target for the circulating hormone ghrelin. In particular, the central ghrelin signalling appears to be of importance for alcohol reward

alcohol to condition a place preference (CPP) in which animals show preference for an alcohol-paired environment, a pivotal test of alcohol reward (Fig. 10.4). Indeed, the GHS-R1A antagonist suppressed the CPP response whether administered during the 4 days of conditioning (in which the animals learn to experience reward from alcohol in one of the two environments) or on the test day (when they may select their preferred compartment in the absence of alcohol). The exact circuitry through which ghrelin modulates alcohol intake as well as alcohol-induced reward in rats remains to be further elucidated, but likely involves actions at the level of the VTA and LDTg [4] rather than the hypothalamus [40].

A Role of the Central Ghrelin Signalling System in Alcohol Dependence

A number of clinical investigations have assessed plasma ghrelin levels in relation to alcohol consumption and alcohol dependence. In healthy individuals alcohol appears to suppress ghrelin secretion, reflected by the reduced blood concentrations that persist several hours after the consumption of alcohol per kg body weight in healthy volunteers [41–43]. This also appears to be the case in alcohol-dependent individuals, as both ghrelin levels and fundic ghrelin production are suppressed

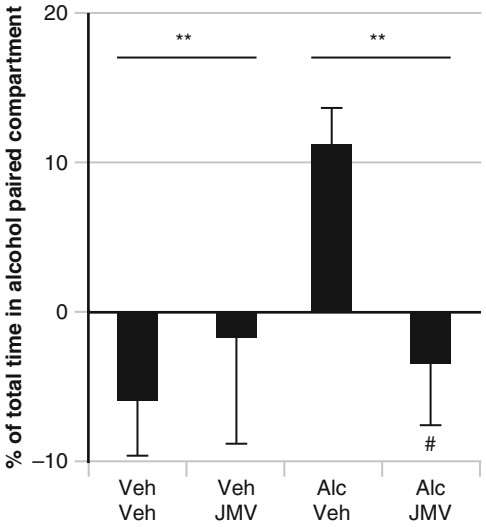

Fig. 10.4 The ability of alcohol to condition a place preference was abolished by peripheral administration of a ghrelin receptor antagonist (JMV2959), given during acquisition of the CPP response. The procedure consisted of preconditioning (day 1), conditioning (days 2–5) and post-conditioning (day 6). On day 1 (preconditioning), mice were injected i.p. with vehicle and initial place preference was determined during 20 min to determine which of the two compartments could be labelled "least preferred" for each mouse. Mice were randomized and received a total of two i.p. injections of alcohol per day in the morning and vehicle conditioning in the afternoon, or vice versa. The mice were placed in the appropriate compartment after injections. On day 6, mice were given free access to both compartments for 20 min. Mice were acutely injected with the GHS-R1A antagonists (JMV2959, i.p.) or vehicle prior to the test session. In a separate series of CPP experiments, NMRI mice were treated with either vehicle or GHS-R1A antagonist (JMV2959, i.p.) prior to the alcohol injection on each conditioning day. In control experiments, vehicle or GHS-R1A antagonist (JMV2959, i.p.) was administered prior to vehicle. On the test day (day 6), the NMRI mice received no treatments and were given free access to both compartments for 20 min. Adapted from Jerlhag et al. (2009) (Proc Natl Acad Sci USA. 106:11318–23) with permission

during periods of active drinking [44–46]. In line with our hypothesis that ghrelin increases the motivational incentive value of alcohol, in alcohol-dependent individuals ghrelin levels are increased during periods of withdrawal and in association with alcohol cravings [44, 46–48]. Kraus and colleagues reported a positive correlation between plasma ghrelin levels and duration of abstinence [46]. Conceivably, the ghrelin signalling system may be especially important for certain subtypes of alcohol-dependent individuals, especially those in which alcohol craving is a major component. In support of a role for ghrelin in alcohol reward are the recent preclinical data showing that the alcohol-induced locomotor stimulation, accumbal dopamine release and CPP are reduced in ghrelin knockout mice [49]. Another study, however, reported that plasma ghrelin levels were only increased during the initial phase of abstinence and did not differ from control subjects at the 3-week time point [48].

Genetics may to some extent explain the development of the complex disease, alcohol dependence. As suggested above, ghrelin production and signalling, including GHS-R1A, might be linked to alcohol dependence. Previously, single nucleotide polymorphisms (SNPs) in genes coding for ghrelin and GHS-R1A have been associated with eating disorders including binge eating disorder [50, 51] and obesity [52]. Therefore, a number of studies have investigated the ghrelin axis in the aetiology of addictive behaviour disorders. Initially, one SNP in the GHS-R1A gene was associated with high alcohol consumption as well as another SNP of the GHS-R1A gene. Also several common haplotypes in both the pro-ghrelin and GHS-R1A genes were associated with an increase in weight and BMI in heavy alcohol drinkers, specifically in non-smoking heavy alcohol drinkers [53]. Furthermore, haplotypes in the GHS-R1A or pro-ghrelin genes are linked with type-2 alcohol dependence or paternal history of alcohol dependence in severely alcohol-dependent females [35]. Given that paternal alcohol dependence is regarded as one of the strongest risk factors for developing the disease [54], it may be suggested that some of the heritability of alcohol dependence may be mediated via polymorphisms in the ghrelin signalling system genes. Supportively, the type 2 subtype of alcohol dependence, associated with polymorphisms in the ghrelin signalling, depends more on genetic risk factors than the type 1 subtype [55]. Even though genetic variability of the ghrelin signalling system may not be considered as a diagnostic marker for alcohol use disorder, it most likely plays a role in the development of addictive behaviours.

A Role of the Central Ghrelin Signalling System in Addictive Behaviour Disorders Other than Alcohol Dependence

The emerging neurobiology of central ghrelin signalling indicates that it may serve as a common denominator to enhance motivated behaviour for natural and artificial reward. This hypothesis is substantiated further by human functional magnetic resonance imaging in which ghrelin administration to healthy volunteers altered the brain response to visual food cues in areas such as the ventral striatum [56], a target area for psychostimulant drugs [10].

Ghrelin stimulates food intake even in satiated animals, indicating that it has a role in non-homeostatic feeding. Indeed, it now seems clear that the suggested physiological role of ghrelin as a circulating hunger hormone [3] likely reflects, at least in part, direct actions within the reward circuits. The first indication that the reward system is a relevant target for food intake was provided by Naleid and colleagues, who showed an increase in chow intake following direct administration into the VTA or the NAcc [57]. Direct administration of a GHS-R1A antagonist into the VTA has been shown to block the orexigenic effects of exogenous ghrelin [16] indicating that circulating ghrelin accesses the VTA for its orexigenic effects. A number of recent reports indicate that central ghrelin signalling at the level of the reward system is required for the intake of rewarding food as

well as for food reward [33, 38, 58–60]. Thus, GHS-R1A emerges as a potential therapeutic target to suppress food reward and motivated behaviour for food, with possibilities for treating eating disorders that lead to obesity, especially those for which a dysfunctional reward system has been implicated (e.g. binging behaviour, compulsive overeating).

Given ghrelin's effects on food intake and food reward, a relevant question is whether the increased consumption of alcohol in mice treated centrally with ghrelin could be driven by the caloric content of the alcohol rather than its incentive rewarding value. Although impossible to rule out, this explanation seems unlikely for several reasons. Firstly, it would be difficult to explain the effects of pharmacological suppression of GHS-R1A in the ability of alcohol to condition a place preference that is clearly independent of its caloric value. Moreover, one of the GHS-R1A antagonists used to assess the importance of GHS-R1A for alcohol reward, BIM28163 [61], achieved a decrease in alcohol consumption but paradoxically increased food intake. Also, peripheral ghrelin has recently been shown to enhance sweet taste independently of caloric content [59].

It seems clear that the mesoaccumbal dopamine system provides a key target for all addictive drugs [11, 62, 63]. Given that ghrelin targets this system, the possibility emerges that the central ghrelin signalling system is of importance for reward from addictive drugs other than alcohol. Ghrelin has been shown to enhance the effects of cocaine on locomotor stimulation and on the ability of cocaine to condition a place preference and also to induce cocaine-seeking behaviour [64–66]. Consistent with this, we found that ghrelin antagonists suppress the locomotor stimulatory and dopamine-releasing effects of cocaine and also suppress its ability to condition a place preference [67]. In this study, we also assessed amphetamine's rewarding effects in these models and, as for cocaine, all of its effects on the aforementioned parameters were suppressed by a GHS-R1A antagonist. In accordance with these data, food restriction, that increases ghrelin levels [68], has been shown to augment cocaine—as well as amphetamine-induced locomotor stimulation—to facilitate the acquisition of cocaine-seeking behaviour and to enhance self-administration of cocaine or amphetamine in rats [69, 70]. It appears therefore that different psychostimulant drugs, operating via different and overlapping mechanisms at the level of the reward system, require central ghrelin signalling (albeit to differing degrees) for their rewarding properties. This further supports the idea that GHS-R1A represents a common denominator in the reward mechanism, providing opportunities for therapy for diverse addiction behaviour disorders.

Pharmacological Suppression of Ghrelin-Responsive Reward Circuits for Addictive Behaviour Disorders

Another interesting aspect regarding future therapeutic strategies for addictive behaviour disorders involving the central ghrelin signalling system is that GHS-R1A appears to be constitutively active in the absence of ligand [71, 72]. Thus, it

is possible to pharmacologically suppress the brain pathways through which ghrelin operates that include a key reward circuit implicated in reward from chemical drugs such as alcohol, independently of ghrelin. Moreover, inverse agonists for GHS-R1A are especially interesting from a drug development point of view, because they offer the possibility to directly inhibit or dampen the ghrelin signal without completely blocking it.

To date, there are no published data for clinical trials with GHS-R1A antagonists or inverse agonists. If, indeed, the afferent gut–brain signal provided by ghrelin is of importance for alcohol cravings in alcohol-dependent patients, especially during periods of withdrawal, it will be interesting to discover whether such future drugs can be used to suppress craving and even prevent relapse.

To date, there are no published reports in which GHS-R1A antagonists or inverse agonists have been tested in man. A number of GHS-R1A antagonists have been tested in preclinical models, mostly in the context of food intake and appetite control [73, 74]. Many of our studies have used the non-peptide antagonist, JMV2959, that has a trisubstituted 1,2,4-triazole structure [75]. In addition to the aforementioned studies on chemical drug reward, including alcohol, we found that this compound is able to suppress ghrelin's acute effects on food intake [76] and also the effects of chronic central ghrelin treatment on fat accumulation [77]. Thus, the compound clearly interrupts ghrelin's central orexigenic and pro-obesity effects, exerted via the ghrelin receptor GHS-R1A. We found that chronic central treatment with this antagonist had no effect itself, however, on body weight or on body fat accumulation in rats fed normal chow [77]. However, in another study we found that chronic peripheral treatment with JMV2959 to mice altered food preference, whereas normal chow food intake was unaffected; we did observe an effect of the antagonist to suppress the intake of a caloric "rewarding" chocolate drink [58]. Collectively these data suggest that GHS-R1A antagonists may be able to selectively suppress the intake of palatable food (that is driven by the incentive value of the food) without affecting normal chow intake (presumed to reflect homeostatic feeding). These findings could be of importance for the therapeutic development of GHS-R1A antagonists, not only for problematic overeating but also for addressing potential concerns of weight loss should these compounds become a new therapy for other addictive behaviour disorders such as alcohol dependence.

Another potential concern for a new therapy that targets the reward system is anhedonia (i.e. reduced liking or pleasure from rewards). According to the incentive salience theory of reward "liking" (emotional/hedonic component) and "wanting" (incentive motivation) represent distinct neurobiological phenomena [12]. To date, there are no publications providing direct evidence that the central ghrelin signalling system is important for hedonic aspects of the reward mechanism, either for natural rewards or for chemical drug reward. Rather, as already discussed, the target reward circuit for ghrelin is intimately associated with incentive motivation, involving the mesoaccumbal dopamine system. One potential pharmacological strategy for avoiding anhedonia would be to use GHS-R1A inverse agonists that would be expected to dampen ghrelin's effects on the dopamine system, without completely blocking them.

Other opportunities for new therapies for addictive behaviour disorders that exploit the central ghrelin signalling system could include agents that interfere with cholinergic or glutamatergic signalling onto the ghrelin-responsive circuits, as already discussed. Another interesting candidate system is the dopamine receptor D1, given that receptor dimerizes with GHS-R1A in vitro [78]. A considerable literature exists in support of a role for the D1 receptor in alcohol intake, preference and oral self-administration in rodents [79, 80], although it is not yet known how agonists and antagonists for this receptor impact upon central ghrelin signalling via GHS-R1A and, hence, ghrelin's effects in vivo. Thus, the physiological relevance and pharmacological consequence of this receptor dimerization remain to be determined. An important aspect of future research in this area could include the development of molecular targets both upstream and downstream of GHS-R1A, given that ghrelin-responsive circuits appear to play an important role in the reward mechanism and, hence, alcohol dependence.

Conclusions

Addictive behaviour disorders encompass a diverse disease area that include substance use disorder and even eating disorders that lead to obesity. With new perspectives and knowledge, the general idea of addiction as substance dependence has changed. Clinical studies of patients with aberrant eating behaviour have shown behavioural parallels between compulsive overeating and chemical addictions (e.g. nicotine, alcohol and psychomotor stimulants) [81]. Additionally, it has been shown that food, when consumed in excess and over time, can cause the same brain neuro-adaptations as drug abuse [82]. It has therefore been suggested that brain functions can be similarly derailed by natural rewards and drugs of abuse. "Behavioural" addictions, such as compulsive overeating, gambling and compulsive shopping, have therefore been included in the definition of addiction and are together with drug dependence called addictive behaviours. Interestingly, human imaging studies have revealed that there is an underlying disruption in the reward systems in the brain [83–86], as well as in brain regions important for inhibitory control [86] in addictive behaviours.

The ghrelin receptor, GHS-R1A, has emerged somewhat unexpectedly as a new and relevant target for this disease area that encompasses diverse addictive behaviour disorders. Future antagonists and inverse agonists that target this receptor will be especially interesting to pursue, given that they target the brain orchestra for which alcohol is a guest conductor that includes the midbrain dopamine system, a system intimately involved in motivated behaviour for both natural and artificial rewards. Although GHS-R1A antagonists and inverse agonists have not yet entered clinical testing for addictive behaviour disorders such as alcoholism, there is good reason for optimism, based on an increasing literature showing beneficial effects of suppressing the central ghrelin signalling system on reward from alcohol [4], cocaine, amphetamine [67] and palatable/rewarding food [38, 58, 60, 88, 89].

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Chapter 11
Ghrelin Receptor Agonists in Cachexia of Human Aging

Ralf Nass and Michael O. Thorner

Abstract Frailty is one of the key features of aging and it is associated with a loss of function, an increased risk of falls, and ultimately loss of the ability to live independently. Based on the demographic shift of the older population, as a result of the aging of the baby boomers, interventions to slow or delay the development of frailty will be of increasing importance. One of the physiologic correlates of frailty is the cachexia of aging. The underlying mechanism of the cachexia of aging is cytokine-associated wasting of protein and energy stores and ghrelin receptor agonists target some of these mechanisms. Clinical trials have shown that ghrelin receptor agonists have orexigenic effects, enhance GH secretion, and increase lean body mass and limb fat mass in the elderly. The potential benefits of ghrelin receptor agonists in the treatment of cachexia of aging and of cachexia secondary to various diseases are reviewed.

Introduction

The increase in life expectancy observed since the end of World War II makes the loss of function of aging increasingly important. In the developed world, people over the age of 80 are the fastest growing subset of the population [1]. The worldwide population aged >65 years is projected to increase from about 249 million in the year 2000 to an estimated 690 million in the year 2030. In 2050, individuals aged 60 years and older will represent 25% of the world's population [2, 3].

R. Nass, M.D.
Department of Internal Medicine, Division of Endocrinology, University of Virginia, University Hospital, Charlottesville, VA, USA

M.O. Thorner, M.B., B.S., D.Sc. (✉)
Department of Medicine, University of Virginia, 450 Ray C Hunt Drive, Charlottesville, VA 22908-1411, USA
e-mail: mot@virginia.edu

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Currently about 7 million Americans over the age of 65 years depend on others for help with some basic task of daily living [4]. Third National Health and Nutrition Examination Survey (NHANES III) data show that 23% of people aged 80 years and older are unable to prepare their own meals and 17% are unable to walk. It is estimated that by 2050 there will be more than 4.5 million hip fractures annually [5] and complications resulting from falls are the sixth leading cause for death in people over 65 years [6]. Currently the chance of dying without disability at age 80 years and older is less than 25%, even for those who are still fully independent at age 65 years [7]. Frail older adults are less able to tolerate the stress of medical illness, hospitalization, and immobility [8]. Strategies to prevent and/or slow the development of physical frailty will become increasingly important in the ensuing decades to enable a higher proportion of the elderly population to maintain independence. The clinical pathway to physical frailty includes anorexia, sarcopenia, and cachexia. This chapter reviews different ghrelin receptor agonists and their potential effects on these physiologic correlates of aging.

Cachexia of Aging

The current literature suggests that frailty, a key feature of aging, is a definable clinical state [9, 10] and is present in elderly subjects who have no overt disease [11]. Frailty is associated with an age-dependent loss of function, loss of physiologic reserve, and increased risk of falls, disability, institutionalization, and ultimately death. One of the physiologic correlates which result in frailty is a phenomenon, called the cachexia of aging, which is upstream in the pathway leading to frailty [12]. Besides the aging process, cachexia is also associated with conditions such as AIDS, heart failure, kidney failure, and cancer and it is also thought to be partially responsible for the weight loss in the elderly [12].

Currently there is no standard definition of cachexia in the elderly. Criteria used to clinically define cachexia include the unintentional weight loss of more than 5% body weight, a BMI of less than 20 kg/m^2 under the age of 65 years and a BMI of less than 22 kg/m^2 at the age of 65 years and older, an albumin level of less than 35 g/L, a low fat-free mass (lowest 10%), and evidence of cytokine excess (e.g., elevated C-reactive protein) [11]. The underlying mechanism of cachexia is a cytokine-associated wasting of protein and energy stores, which results in an equal loss in fat and muscle mass. Opposite to starvation, resting energy expenditure, protein degradation, serum insulin levels, and serum cortisol levels have been shown to be increased in cachexia [13]. Cachexia is associated with higher than normal concentrations of tumor necrosis factor alpha, interleukin (IL) 1, IL-6, serotonin, and interferon γ; these proinflammatory cytokines play an important role in cachexia of cancer and AIDS. Similar cytokine elevations are found during the cachexia of aging [14]. The cachexia-related increase in cytokines has been shown to have a significant impact on the balance between muscle protein synthesis and muscle protein breakdown and is mediated by induction of ubiquitin-dependent proteolysis

[15]. In clinical studies, IL-6 has been shown to be elevated in community-dwelling frail older adults and is associated with sarcopenia and weight loss [16]. Cappola et al. [17] found that the combination of low IGF-I and high IL-6 levels confers a high risk for progressive disability and death in older women. One of the key features of the cachexia of aging is the involuntary loss of body weight.

Weight Loss in the Elderly

Involuntary weight loss in the elderly aged 60 years and older is predictive of mortality. Rosenbaum et al. [13] showed that the 95% CI over 6 months for change in body weight in healthy adults was ±5%. While rates of change beyond the 5% limit can be considered abnormal, rates of change within these limits can also be abnormal if changes within different body compartments show opposite changes. Nursing home residents who have continued weight loss have a 30% chance of death in the following 6 months, while in those who gained weight, the likelihood of dying is reduced to 10% [18]. Several studies suggest a relationship between low BMI (kg/m^2) and increased death rate in the elderly. At a BMI of less than 20.5 in men >75 years of age, a 20% higher mortality risk is observed, while in women >75 years of age a 40% higher mortality risk has been described [18]. Unintentional weight loss has also been associated with a greater rate of functional decline [19] in community-dwelling older adults and it is thought to be a predictor of mortality in Alzheimer's disease.

Sarcopenia in the Elderly

The age-associated muscle loss, also called sarcopenia, is one of the major contributing components of the multi-systemic decline leading to loss of function. The age-dependent muscle loss has been linked to physical frailty and decreased capacity for independent living. The etiology of the age-dependent muscle loss includes a wide variety of factors, one of them being the age-dependent increase in catabolic cytokines, such as interleukin-6 and TNF-α [20]; these cytokines are considered to be a central part of the underlying mechanism of the cachexia of aging, leading to a decrease in muscle protein synthesis and an increase in muscle protein degradation.

Ghrelin Receptor Agonist

Physiologic Effects of Ghrelin Receptor Agonists

Increased protein breakdown and increased energy expenditure are key mechanisms of cachexia. Ghrelin receptor agonists target both of these mechanisms by decreasing energy metabolism, antagonizing protein breakdown, slowing energy expenditure,

Table 11.1 Physiologic effects of ghrelin receptor activation

Increase in appetite
Increase in food intake
GH release
Increase in lean body mass
Increase in gastric motility
Increase in limb fat mass

and increasing circulating GH levels which have been shown to decrease protein degradation. The story of ghrelin is one of reverse pharmacology [21]. The first ghrelin receptor agonists were described in the 1970s by Bowers et al. [22], about a quarter of a century before the ghrelin receptor and the ghrelin peptide were discovered. Bowers proposed that opiates stimulate GH release. He and his colleagues argued and then demonstrated that analogs of enkephalin could be developed to selectively stimulate GH release without their analgesic or addictive properties [23]. The initial research focused on the GH releasing effect of these peptides. In the 1990s the first orally available ghrelin receptor agonists were tested [24] and were named GH secretagogues. In 1996 the GH secretagogue receptor, later shown to be the ghrelin receptor, was expression cloned from the pituitary [25] and in 1999 the natural ligand of the receptor, named ghrelin, was discovered using a cell line expressing the ghrelin receptor [26]. Ghrelin is a 28 amino acid containing peptide with acylation of the third amino acid, a serine; this modification is unique and is mandatory for its biologic effects at the receptor. Ghrelin is present in two major forms in the circulation, an acylated and an unacylated form [27]. Only the acylated form has been shown to activate the ghrelin receptor. Experiments of the structure–activity relationship of ghrelin [28] showed that a bulky lipophilic residue at Ser^3 residue of ghrelin is necessary for its affinity towards the GHS-R1a receptor. The n-octanoyl group can be replaced by a decanoyl or a palmitoyl group as well as a benzoyl or an adamantyl group. The studies also showed that the ester function at Ser^3 can be replaced by an amide function without loss in biological activity and that the four N-terminal residues of ghrelin are necessary for binding and activation of the GHS-R1a receptor [29, 30]. Table 11.1 summarizes some of the main physiological effects thought to be mediated by acyl-ghrelin. The orexigenic, adipogenic, and GH releasing effects of ghrelin [31, 32] could be predicted based on the early results of the experiments performed with GH secretagogues [33, 34] which are now called ghrelin receptor agonists. The discovery of ghrelin enabled the study of the regulation and distribution of ghrelin [35, 36], as well as potential age-dependent changes of circulating ghrelin levels. While initial studies with ghrelin knockout and ghrelin receptor knockout animals did not result in a definitive phenotype [37], more recent studies by Zhao et al. [38] suggested that ghrelin might be necessary for triggering the GH response to prolonged nutritional deprivation that prevents hypoglycemia under conditions of long-term caloric restriction [39]. This seems not to be the case during complete caloric restriction such as prolonged fasting where circulating acyl ghrelin levels have been shown to decline [40].

Several studies have addressed the effects of ghrelin on cytokines. Animal studies by Dixit et al. [41] showed that ghrelin treatment of rodents reduces LPS-induced chronic production of pro-inflammatory cytokines such as IL-6, TNFα, and IL-1β, an effect which is thought to be mediated through the GHSR1a receptor. In vitro studies in endothelial cells suggest that ghrelin is able to inhibit proinflammatory responses and NFkB activation [42]. Cachexia, anorexia, as well as sarcopenia are age-associated conditions, ultimately leading to frailty. The strong orexigenic and GH releasing effects of ghrelin and ghrelin receptor agonists make these compounds potential candidates for the treatment of these conditions. This assumption is supported by several studies which showed an age-dependent decline of total ghrelin [43] and acyl-ghrelin using 24-h frequent sampling [44].

Clinical Studies with Ghrelin Receptor Agonists in the Elderly

MK677. The number of clinical studies with ghrelin mimetics, some of them orally active, has been relatively small, when compared to the number of studies testing ghrelin and its beneficial effects in treating cachexia associated with cardiac failure, COPD, cancer, and ESRD [45, 46]. This is especially astonishing as some of the ghrelin mimetics are orally available and once-daily administration allows for a duration of effect of 24 h, while ghrelin has to be given subcutaneously or IV and has a short estimated half-life of about 15–20 min [47]. One of the first studies to address the effects of a ghrelin receptor agonist, MK-677, under catabolic conditions was published by Murphy et al. [48]. MK-677, a nonpeptide spiropiperidine, was developed by Merck and it was initially used to expression clone the GHS receptor (GHS-R) [33]. The use of MK-677 was of interest as it is orally active and it enhances the preexisting pulsatile GH release [33]. The study showed that administration of MK-677 improved the nitrogen balance response to 2 weeks of caloric restriction [48]. Chapman et al. [49] showed in a 4-week double-blind placebo-controlled study that once-daily oral MK-677, given to healthy older adults, enhances pulsatile GH release and IGF-I concentrations to those seen in young adults. Eight weeks' treatment of obese males with MK-677 resulted in a decline in the LDL/HDL ratio [50] and in an increase in fat-free mass. In a multicenter, randomized, double-blind placebo-controlled 18-month study in 292 postmenopausal women, MK-677 given in combination with alendronate increased BMD at the femoral neck vs. alendronate alone; however this effect was not seen for the lumbar spine, total hip, or total body when compared to alendronate alone [51]. The authors concluded that while MK-677 in combination with alendronate has significant positive effects on the bone formation at the femoral neck, the lack of enhancement at other sites made it a less attractive choice when compared to alendronate alone [51]. Bach et al. [52] showed that MK-677 when given to hip fracture patients after hip surgery in a group of patients aged 65 years and older resulted in a greater improvement relative to placebo in three of four lower extremity functional performance measures. However the study did not find a statistically significant effect when

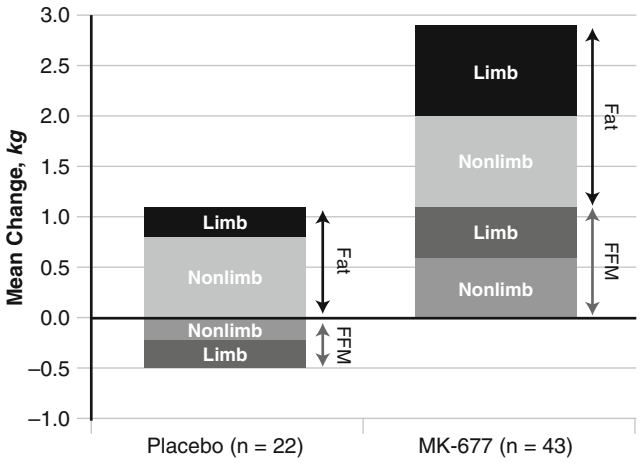

Fig. 11.1 Mean changes in fat and fat-free mass (FFM) at 12 months treatment with MK-677 or placebo in healthy older adults [53]. Limb = appendicular lean soft tissue and appendicular fat; nonlimb = total minus limb

measuring the ability to live independently or in the domain of the Sickness Impact Profile for Nursing Homes (SIP-NH) 6 weeks after surgery. The longest study to date to assess the effects of MK-677 in healthy older adults was published in 2008 [53]. The study showed that one-year treatment with MK-677 increases fat-free mass by 1.1 kg while the placebo group had a 0.5 kg muscle loss during the same time period. The effect was sustained in the group which received the ghrelin receptor agonist for a total of 2 years. No significant increase in total body fat was found; however there was a significant increase in subcutaneous limb fat (see Fig. 11.1). The changes in muscle mass did not translate into changes in muscle strength or function. The study included mainly physically fit, active, healthy older adults; this probably explains the discrepancy between the increase in muscle mass without a change in function and strength. Further the subjects were not participating in supervised exercise programs.

Capromorelin. Another ghrelin receptor agonist, which was tested in healthy older adults, is the pyrazoline–piperidine capromorelin. In a 1-year study, capromorelin increased lean body mass by 1.4 kg vs. 0.3 kg in the placebo group. The treatment group was a group of older adults with mild functional limitation. The group also found an improvement in tandem walk and stair climb. However the analysis required the pooling of the treatment group, which had different dosing groups [54].

Other Ghrelin Receptor Agonists Used Outside the Aging Field

In the past 15 years several other ghrelin receptor agonists have been developed and tested, either to be used as a diagnostic tool in GH deficiency or as a therapeutic agent mainly in the field of cachexia (pulmonary, cardiac) and gastric motility.

RC-1291. Oral administration of the ghrelin receptor agonist RC-1291 resulted in a dose-related increase in body weight in healthy volunteers [55] and studies in cancer patients (predominantly lung cancer) showed a small increase in lean body mass [56].

ARD-07. ARD-07, a hexarelin analog, is an orally available synthetic ghrelin receptor agonist which has proven GH releasing effects in healthy adults; however data about its long-term use in humans have not been published [57, 58].

TZP-101. The IV ghrelin agonist Ulimorelin (TZP-101), a macrocyclic peptidomimetic, has been studied in patients with diabetic gastroparesis and postoperative ileus and showed positive effects on nausea associated with gastroparesis [59].

NN703. The orally active ghrelin mimetic NN703, an acylated dipeptide, which is orally available, showed a significantly increased IGF-I and GH levels, when given over 7 days [60], but no long-term data have been published to date. The compound is being tested to be used for the diagnosis of GH deficiency.

SUN11031. Synthetic ghrelin SUN11031 showed an improvement in lean body mass and function using SPPB in COPD patients with cachexia (20–40% of COPD) [61].

Besides these ghrelin mimetics, which have been tested in clinical trials, several other analogs have been developed, but have not been tested in clinical studies, i.e., no published results are available [28].

Potential Adverse Effects and Limitations in the Use of Ghrelin Receptor Agonists for the Cachexia of the Elderly

Cancer Risk

One of the main concerns about a therapy, which increases GH and IGF-I, is the potential risk of developing cancer or cancer progression of preexisting occult tumor cells. IGF-I has anti-apoptotic effects leading to proliferation of almost all tissues. This might be especially a concern in the older population with an increased number of genetically damaged cells. Previous studies had suggested that increased IGF-I levels are associated with increased cancer risk [62]. The results of more recent studies contradict these earlier conclusions. A recently published study from Amsterdam with 1,273 older persons, who were followed over a 11.6-year follow-up period, showed no association between IGF-I and fatal and nonfatal cancer [63]. Data by Holly et al. in 111,000 men showed that IGF-I is not related to the incidence of PSA-detected prostate cancer [64]. The overall number of studies, as well as the total number of volunteers tested so far, is too low to estimate cancer risk in patients and healthy volunteers treated with ghrelin receptor agonists. Independent of the potential growth promoting effects via the stimulation of the GH/IGF-I axis, direct ghrelin effects on tumor cells need to be considered especially as several tumor cells have been shown to express ghrelin receptors. Long-term studies with a large number of participants are necessary to address this question adequately. To date, the

existing data do not support an increased cancer risk with the use of ghrelin receptor agonists. Koo et al. [65] showed in rodents, inoculated with lymphoid tumor cells, that treatment with the ghrelin agonist MK-677 resulted in a decreased incidence of tumor metastases. The group also found a decrease in tumor progression with the administration of the ghrelin receptor agonist.

Glucose Metabolism

One of the concerns in the use of ghrelin receptor agonists in the elderly includes the potential deterioration of insulin sensitivity especially in elderly with preexisting impaired glucose tolerance. In our study with healthy older adults, treated for 1 year with the ghrelin receptor agonist MK-677 [53], hemoglobin A1c (HBA1c) and fasting glucose levels increased significantly; however this change was small when expressed in absolute terms and the fasting glucose levels remained in the normal range. Similar effects on glucose metabolism were reported by Bach et al. [52] after 6 months of treatment with MK-677 in healthy older adults after hip fracture. White et al. [54] reported the same effect on glucose metabolism with the ghrelin receptor agonist Capromorelin. Svensson et al. [50] did not find an increase in fasting glucose or insulin concentrations after 8 weeks of treatment with MK-677; however the glucose tolerance test showed an impairment of glucose homeostasis. Of note, the synthetic ghrelin receptor agonist SUN 11031 administered to COPD patients with cachexia for 3 months did not result in changes in fasting or post-oGTT glucose or HBA1c [61]. Overall, several of the ghrelin receptor agonist studies showed an increase in glucose levels; however these changes were overall small.

Adipogenic Effect

Results of animal studies suggest an adipogenic effect of ghrelin [31]. Both long-term and short-term studies with ghrelin receptor agonists did not show a change in total or abdominal visceral fat [53, 54, 66] even though limb body fat was increased in one study [53].

Ghrelin Resistance

One of the potential concerns in the use of ghrelin mimetics in catabolic situations, such as cachexia, relates to the presence of ghrelin resistance as shown by Strasser et al. [67]. In this study ghrelin was given IV in two different doses (2 or 8 μg/kg bw) on two different admissions seven days apart to patients with advanced cancer and anorexia/cachexia. While the infusion was well tolerated, no effect on IGF-I levels or the nutritional intake and eating-related symptoms was observed. Opposite to these findings, earlier studies by Neary et al. [68] showed a 31% increase in energy intake after a ghrelin infusion in cancer patients

with anorexia. Ghrelin resistance is also thought to be responsible for the increased circulating ghrelin levels and the lack of response to a ghrelin infusion in anorexia nervosa [69, 70].

GH Releasing Effects in Catabolic Patients

Concerns when using any type of GH-releasing agent in a catabolic situation is based on the findings published by Takala et al. [71], which was conducted in ICU patients. While GH treatment was able to improve nitrogen balance, the treatment was associated with an increase in morbidity and mortality. However the patients treated in this study were severely ill and received very high doses of GH between 0.07 and 0.13 mg/kg bw per day. These patients were in an extreme catabolic situation which is different from the age-associated cachexia. In addition, ghrelin receptor agonists have the potential advantage to enhance the preexisting pulsatile GH release and to leave the physiologic GH pattern intact. In addition they have an inbuilt safeguard in that IGF-I feeds back at the pituitary to prevent overstimulation of the somatotropic axis.

Agents Used in the Age-Associated Weight Loss

The current list of compounds used to treat age-associated weight loss includes megestrol acetate, dronabinol, testosterone, and oxandrolone. Most of them have significant side effects [72–77]. Table 11.2 lists some of the most widely used agents for weight loss in the elderly and compares them with the side effect profile of ghrelin receptor agonists.

Conclusion

The underlying mechanism of the cachexia of aging is thought to be mediated by increased cytokine production. The resulting phenotype is characterized by a decrease in body weight and a loss of muscle tissue and adipose tissue as well as a decline in function and the loss of independence. These changes ultimately lead to frailty.

Ghrelin receptor agonists have the potential to alleviate some of the consequences of the cachexia of aging. Compared to the currently available interventions used for cachexia and unintentional weight loss in the elderly, ghrelin receptor agonists show similar effects, but have fewer adverse effects. Ghrelin agonists address the problem at each level—they reduce cytokine production, increase appetite, enhance GH secretion, and increase lean body and limb fat mass. Further research in this area is mandatory to compare the efficacy and safety of ghrelin receptor agonists to currently used agents in the treatment of the cachexia of aging.

Table 11.2 Side effect profile of ghrelin receptor agonist in comparison to other compounds used for weight loss and muscle loss in the elderly

	Megestrol acetate	Dronabinol	Testosterone	Oxandrolone	Ghrelin receptor agonist
Adrenal insufficiency	+	–	–	–	–
Diabetes mellitus	+	–	–	–	(+)
DVT	+	–	–	–	–
Erectile dysfunction	+	–	–	–	–
CNS side effects	–	+	–	–	–
BPH	–	–	+	–	–
Lipid profile deterioration	–	–	+	–	–
Liver toxicity	–	–	+	+	–
Increased Hct	–	–	+	–	–
G.I symptoms	–	–	–	+	–
Hirsutism (women)	–	–	+	–	–

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Chapter 12
Use of Ghrelin and Ghrelin Receptor Agonists in Cancer- and Chemotherapy-Induced Cachexia

Basil Burney and Jose M. Garcia

Abstract The Cancer Anorexia–Cachexia Syndrome (CACS) is present in up to 80% of cancer patients decreasing their quality of life and survival. Nevertheless, treatments for this condition are lacking. Ghrelin and its mimetics appear promising in the treatment of CACS. Ghrelin increases growth hormone/IGF-1 levels, body weight, and appetite, and decreases energy expenditure in healthy subjects. Also, animal and human studies have shown favorable anabolic effects of ghrelin in the setting of cancer. In preliminary studies, anamorelin, an orally available ghrelin mimetic, also seems to improve weight loss in cancer cachexia patients. Furthermore, in animal models of cisplatin-induced cachexia and emesis, ghrelin infusion stabilizes appetite, increases weight, and improves GI side effects. In several short-term studies ghrelin was found to be safe; however, long-term safety of ghrelin and ghrelin mimetics has not yet been fully established. In summary, more studies are needed looking into the long-term safety and efficacy of ghrelin and its mimetics in cancer patients and other chronic diseases associated with cachexia.

Introduction: Cancer-Induced Cachexia

Cachexia is defined as a hypercatabolic state with a weight loss of at least 5% of the pre-illness body weight. It is characterized by accelerated loss of skeletal muscle with or without loss of fat mass in the context of a chronic inflammatory response [1]. It can occur in the setting of cancer as well as any chronic disease including

B. Burney, M.D.
Department of Endocrinology, Michael E. DeBakey VA Medical Center, Houston, TX, USA

J.M. Garcia, M.D., Ph.D (✉)
Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center, Baylor College of Medicine, 2002 Holcombe Blvd, Houston, TX 77030, USA
e-mail: jgarcia1@bcm.edu

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chronic infections such as AIDS, chronic obstructive pulmonary disease, heart failure, chronic liver disease, and rheumatoid arthritis. The incidence of cachexia depends among other things on the tumor site, ranging from 40% in breast cancer to more than 80% in gastric, esophageal, and pancreatic cancer [2, 3]. Eventually, cachexia along with anorexia, defined as a decrease in appetite and food intake, is present in up to 80% of terminally ill patients with cancer and may be the direct cause of death in some of them [4].

Pathogenesis

The profound weight loss suffered by patients with cancer cachexia is due to a combination of decreased energy intake and increased resting energy expenditure (REE) [5]. In addition, abnormalities of carbohydrate, protein, and fat metabolism lead to continued mobilization, utilization, and ineffective repletion of host tissue, despite adequate nutritional support. This weight loss cannot be treated adequately with aggressive feeding. In cancer cachexia, patients tend to lose both lean body mass as well as fat mass. However, the loss of lean body mass, most notably skeletal muscle, is more prominent than the loss of fat mass. This is in contrast to simple starvation, which is characterized by caloric deficiency that can be reversed by increasing food intake and where most of the weight loss is due to fat loss [6]. Multiple factors have been implicated in the pathogenesis of cancer cachexia. These patients have increased muscle proteolysis [7], increased adipose tissue lipolysis [8], and increased REE [9]. All of these metabolic changes result in loss of both lean body as well as fat mass. The main factors contributing to these changes are discussed below.

Anorexia

Up to 80% of patients with advanced cancer experience a decrease in appetite and food intake [10, 11]. This is caused by the tumor itself since it is present even in treatment-naïve patients but it is usually worsened by the administration of chemotherapy or radiotherapy that may cause inflammation, nausea, vomiting, alterations in taste and smell, and gastroparesis [12, 13]. Cancer cachexia patients appear to maintain the same relative food preferences but consume all foods in lesser amount [11].

Inflammation and Increased Energy Expenditure

An increase in energy expenditure has been described in cancer patients [9]. This partly explains the lack of effect of nutritional interventions alone in cachexia as opposed to simple starvation where energy expenditure is usually decreased. Multiple inflammatory cytokines play an important role in the pathogenesis of

weight loss in cancer patients. It has been suggested that tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta, and IL-6 contribute to the weight loss by increasing REE [14, 15] and by inducing anorexia [16]. Also, it has been shown in mouse models that IL-6 administration results in reduction of body weight, increasing lipolysis and suppressing appetite [17, 18]. Similar results were obtained in another mouse model in which intraperitoneal injection of IL-1 beta decreased food intake and induced weight loss [19]. There have been multiple studies involving cancer cachexia patients that have detected elevations in peripheral blood mononuclear cell cytokine concentrations, especially TNF-alpha and IL-6 [20, 21] supporting the role of inflammation in this setting.

Decreased Anabolism

A number of studies in cachectic subjects also have shown that certain anabolic factors that play an important role in the development and maintenance of muscle mass and function are decreased in this setting. These include downregulation of the growth hormone (GH)-insulin-like growth factor-1 (IGF-1) [22] axis and testosterone [23]. However, the extent to which these abnormalities contribute to cancer cachexia remains uncertain.

Other Factors

In addition, multiple nonspecific factors related to cancer itself or the treatment of cancer further contribute to the decreased nutrient intake or absorption and thereby contributing to cachexia. These include dysphagia, malabsorption resulting from tumor invasion of the gastrointestinal tract or intestinal resection, early satiety due to abdominal mass, enlarged liver (metastasis), enlarged spleen (in cases of leukemia), or ascites. Excessive sedation, nausea, and constipation are common side effects of opioids and can also contribute to a decrease in food intake and weight loss in these patients.

Treatment of Cancer Cachexia

The optimal therapy for anorexia and cachexia is curing the underlying cancer [24]. Unfortunately, this goal is often not attainable with currently available treatments. In the past, cachexia and anorexia were seen as unavoidable consequences of cancer or as side effects of its treatment. However, in recent years special emphasis has been placed on how to reduce the symptom burden in this population.

Anorexia is a common factor identified with weight loss in cancer patients. Thus, therapeutic strategies that improve appetite are a reasonable first step for patients experiencing weight loss. However, due to the involvement of multiple metabolic as well as pro-inflammatory mediators in the pathogenesis of cancer-induced cachexia, treatment of anorexia alone is not sufficient to prevent or treat the disease. As an example, trials using hypercaloric feeding improved body weight but were not shown to increase lean body mass or survival [6, 25].

An integrated approach to treat cancer anorexia and cachexia including both nutritional counseling and pharmacotherapy has been proposed using appetite stimulants or orexigenic, anti-catabolic, anti-inflammatory, and anabolic agents (primarily hormonal). Corticosteroids and progestational agents are used extensively off-label for the treatment of cachexia in the setting of cancer. However, these drugs do not appear to improve survival, nor have they been shown to improve global quality of life in the majority of studies that have examined such parameters. Progestational agents such as megestrol acetate and medroxyprogesterone acetate have long been used as the first-line pharmacological therapy for cancer anorexia and cachexia [26] in spite of not being approved in the USA for this particular indication. They are effective in improving food intake, and to a lesser extent body weight. The body weight gain induced by these agents is mainly due to water retention and some increase in fat mass with no effect on skeletal muscle mass. Furthermore, they should be used with caution as their use may lead to deep venous thrombosis, adrenal insufficiency, vaginal spotting, and sexual dysfunction.

Corticosteroids were one of the first pharmacological agents used for the treatment of cancer cachexia. The exact mechanism by which steroids exert their beneficial effects in this patient population is not entirely known. However, it has been suggested that their orexigenic properties are related to their euphorigenic and anti-inflammatory effects. Corticosteroids do not cause weight gain in cancer cachexia patients but they have shown limited effect of up to 4 weeks on symptoms such as appetite, food intake, sensation of well-being, and performance status [27]. Although corticosteroids seem to improve the quality of life of terminal cancer patients and can be used as palliative therapy, unfortunately their use does not seem to have any significant effects in the reduction of mortality. Despite a wide range of adverse effects associated with steroids including weakness, delirium, osteoporosis, adrenal insufficiency, and immunosuppression, the use of corticosteroids in the cancer population is very popular primarily due to their symptomatic benefits.

Several other classes of drugs that theoretically would benefit patients with cancer cachexia have been investigated or are currently under investigation. Cannabinoids that have shown to benefit HIV-associated cachexia patients failed to demonstrate prevention of weight loss in patients with cancer cachexia [28]. Similarly another orexigenic agent, cyproheptadine which has anti-histaminic as well as anti-serotoninergic properties, failed to show weight-related benefits in spite of mild improvement in appetite [29]. Some anti-inflammatory drugs targeting the TNF-alpha and other inflammatory factors have been studied including eicosapentaenoic acid (EPA), thalidomide, and pentoxifylline. However, studies conducted so far have not shown weight-related or mortality benefit for patients on these

medications [30–33]. A host of other treatments including androgens, nonsteroidal anti-inflammatory drugs, serotonin antagonists, metoclopramide, and insulin have been studied in the past without much success or have involved only a small number of patients [34–38] and are under development.

The development of therapies for the prevention or treatment of cancer-related anorexia and cachexia is desperately needed because there are currently no proven effective treatments for this condition that significantly reduces functional performance and quality of life in this population.

Role of Ghrelin and Ghrelin Mimetics in Cancer Cachexia

One class of agents that appears promising for the treatment of cachexia in animal models and limited human trials is agonists of the growth hormone secretagogue receptor 1a (GHSR-1a), including ghrelin and its mimetics (growth hormone secretagogues or GHS). Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor (AKA ghrelin receptor, GHS-R1a) [39, 40]. It is considered a meal-initiating hormone as its serum levels rise as fasting time increases and then drop after a meal has been consumed [41]. There are two circulating forms of ghrelin: acylated and unacylated (desacyl). Of these, the acylated form is essential for ghrelin's biological activity through the GHS-R1a. This receptor is expressed in a variety of tissues, including appetite-regulating centers in the hypothalamus and brain stem, lymphocytes, and gastrointestinal tract [42–44]. However, it is probably not expressed in striated muscle cells or adipocytes. This suggests that its effects in these target organs are indirect (i.e., centrally mediated through autonomic nervous system efferents, through GH/IGF-1 axis, through its anti-inflammatory effects, etc.) or mediated through a novel, not-yet-identified receptor.

Potential Mechanisms of Action of Ghrelin in Cancer Cachexia

Ghrelin increases GH/IGF-1 levels: Ghrelin plays an important role in a variety of physiological processes. Activation of GHS-R1a by ghrelin or ghrelin mimetics in other settings potently stimulates growth hormone secretion, leading to an increase in IGF-1 levels [45, 46]. Given that cachexia is a state of relative "GH resistance" with high GH levels and low IGF-1 levels [22], restoration of IGF-1 levels could potentially improve host function (see Fig. 12.1).

Ghrelin is a potent orexigenic agent: Ghrelin also plays a role in energy homeostasis by stimulating food intake and promoting adiposity via growth hormone-independent mechanisms. Ghrelin and ghrelin mimetics increase food intake and body weight in non-cancer patients [47, 48]. Reversal of anorexia in the setting of cancer could benefit patients even if this is not sufficient to restore normal body weight.

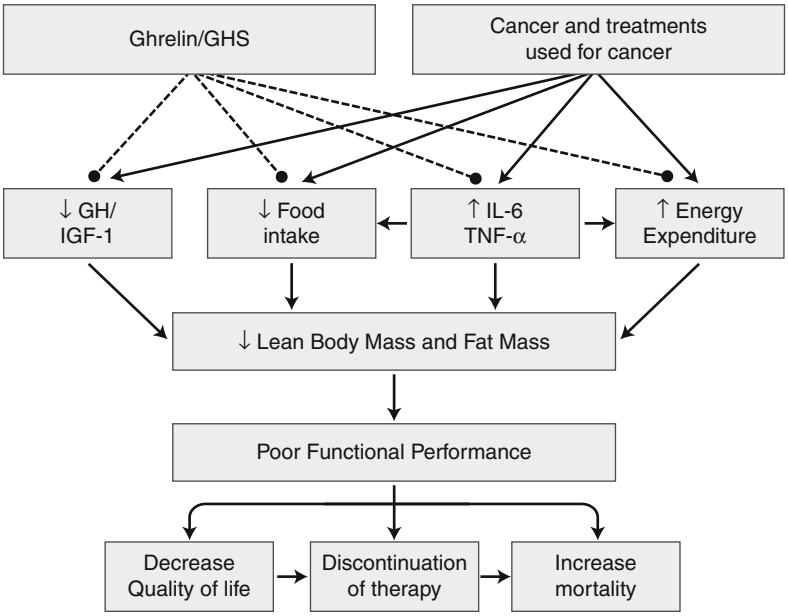

Fig. 12.1 Proposed mechanisms for the effects of ghrelin or ghrelin mimetics (GHS) in cancer-cachexia. Cancer and the treatments used in this setting cause a decrease in anabolic factors such as GH and IGF-1 and in food intake and an increase in inflammation and in energy expenditure. These changes lead to a decrease in lean and fat mass and in functional performance that significantly affect the quality of life and can cause poor tolerance to therapy and increased mortality. Evidence suggests that ghrelin and GHS can increase GH/IGF-1 and food intake and decrease inflammation and energy expenditure, preventing the subsequent changes in body composition, function, and quality of life

Ghrelin decreases energy expenditure: In non-cancer patients, ghrelin mimetics increase body weight and reverse the negative nitrogen balance induced by starvation independently of their orexigenic effects. These findings suggest that ghrelin's effects are not entirely mediated through an increase in appetite and that other mechanisms, such as a decrease in energy expenditure, are involved [49]. Supporting this hypothesis, ghrelin administration was recently found to decrease energy expenditure in non-cancer human models [50].

Ghrelin decreases inflammation: Ghrelin also inhibits production of anorectic proinflammatory cytokines [51, 52]. Thus, ghrelin exhibits anti-catabolic actions through both growth hormone-dependent and -independent mechanisms. The sum effect of ghrelin is an increase in appetite and food intake, which, when coupled with ghrelin-mediated anti-inflammatory activity, have led to the current interest in the use of this agent in human cachexia.

Effects of ghrelin in muscle and adipose tissue: Ghrelin is primarily expressed in the stomach but it is also made in fat and muscle. However, most but not all reports have shown that its known receptor GHS-R 1a is not expressed in these tissues [53, 54]. This suggests that ghrelin's effects in these tissues are indirect or mediated

through a different receptor [55]. Ghrelin reduces fat oxidation and increases adiposity [56]. Kos et al. recently reported that ghrelin increases lipogenesis. However, this effect appears to be mediated through a different receptor [57]. Theander-Carrillo et al. [58] showed that chronic low-dose ghrelin administration induces the mRNA expression of fat storage enzymes and suppresses the expression of the key regulatory enzyme for fatty acid oxidation, carnitine palmytoil transferase-1α (CPT1-α) in white adipose tissue when administered centrally, and that these effects are independent of food intake. Also, low-dose ghrelin administered peripherally was shown to increase lipogenesis by increasing fatty acid synthase (FAS) and to decrease CPT1a mRNA expression in liver [59].

In muscle, upregulation of muscle-specific ubiquitin ligases atrogin-1 and muscle RING finger protein 1 (MuRF1) appears to be critical in the activation of the ubiquitin–proteasome pathway [60]. These ligases can be induced by several mechanisms in the setting of cancer including downregulation of IGF-1 and an increase in inflammatory mediators. Ghrelin mimetics were recently shown to prevent activation of these ubiquitin ligases atrogin 1 and MuRF-1 induced by dexamethasone administration in an animal model [61]. Although the mechanisms leading to these changes have not elucidated, a decrease in inflammation and/or an increase in IGF-1 activation via GHSR-1a-mediated mechanisms could mediate the decreased expression of these ligases. Nevertheless, recent reports suggest that inactive des-acylated ghrelin is active in muscle via another receptor [62].

Ghrelin Levels in Cancer Cachexia

Fasting plasma ghrelin level is inversely related to body mass index (BMI), and its level increases with weight loss induced by caloric restriction [63, 64]. Total ghrelin levels are increased in energy-negative states. For instance, patients with anorexia nervosa exhibit significantly elevated fasting levels of ghrelin that return to normal when body weight is normalized [65]. Individuals with other chronic debilitating diseases like congestive heart failure [66] and COPD [67] are also known to have increased levels of total plasma ghrelin.

With regard to cancer-induced cachexia, ghrelin plasma concentration increased with progression of cachexia in a melanoma cell line tumor model in mice [68]. However, in human cancer subjects, higher, normal, or low levels of ghrelin have been demonstrated. Wolf et al. reported statistically significant elevated levels of ghrelin in 18 patients with breast and colorectal cancer [69]. Shimizu et al. reported 1.3–1.5-fold higher levels of ghrelin in 21 patients with lung cancer-induced cachexia when compared to those without cachexia and healthy controls [70]. Similar results were reported by Malendowicz et al. in 18 patients with prostate cancer when compared to BPH and control patients [71]. In contrast, ghrelin levels were reported as normal in 58 cachectic patients with gastric cancer and 20 patients with colorectal cancer in another study [72]. Interestingly, D'Onghia et al. showed statistically significant lower levels of serum total ghrelin levels in 29 patients with

colorectal cancer when compared to 50 healthy controls [73]. They also showed lower levels of ghrelin in patients with advanced cancer when compared to earlier stages of cancer. However, this study did not comment on the presence or absence of cachexia in these patients and active ghrelin levels were not measured. Our group looked into active ghrelin levels and active-to-total ghrelin ratio in cancer-induced cachexia. We evaluated 21 patients with cancer-induced cachexia, 24 cancer patients without cachexia, and 23 control subjects without cancer. Active ghrelin levels and the active-to-total ghrelin ratio were significantly increased in subjects with cancer-induced cachexia, compared with cancer and non-cancer controls [22]. Cancer-induced cachexia patients had various cancer diagnoses and staging, suggesting that this increase in ghrelin is distinctive to the cachexia syndrome and not restricted to a specific type or stage of cancer. This increase in the active-to-total ghrelin ratio observed cannot be explained simply by an increase in ghrelin secretion and suggests that other mechanisms, such as a decreased inactivation, may also play a role. Also there was no evidence of increased appetite in subjects with cachexia, which was measured by a visual analog scale when compared to the other 2 groups.

Taken together, these results suggested that cachexia could be considered a state of ghrelin resistance as active ghrelin levels are generally increased in spite of the weight loss and anorexia seen. However, new data suggest that this resistance is only partial as administration of ghrelin or ghrelin mimetics can partially reverse or prevent cachexia. The data from these clinical trials are discussed next.

Preclinical Data Supporting Ghrelin Use in Cancer Cachexia

Ghrelin has been studied in different cachexia animal models including CHF, COPD, and chronic kidney disease. So far there have been several animal studies that looked into the role of ghrelin in cancer cachexia. Hanada et al. [74] administered intraperitoneal injections of twice-daily ghrelin in mice implanted with human melanoma cell line which is known to cause severe cancer cachexia. Six days of ghrelin administration increased the body weight of vehicle-treated and tumor-inoculated mice when compared to the corresponding mice that received vehicle. Ghrelin also caused increased food intake in both tumor-inoculated and vehicle-treated mice in comparison to saline-treated mice. There was also statistically significant increase in white adipose tissue in vehicle-treated mice when compared to their saline counterparts. Lastly, there was no evidence of tumor progression or significant increase in tumor size in the group of mice receiving ghrelin.

Wang et al. [75] also studied the role of ghrelin in a mouse model of cancer cachexia. In this study tumor-bearing mice or sham-implanted mice were intraperitoneally administered with twice-daily dose of either 20 mcg/day (low dose) or 40 mcg/day (high dose) of ghrelin or saline for 10 days. Results showed that ghrelin treatment increased food intake effectively in both low dose as well as high dose in non-tumor-bearing mice, while only the high dose of ghrelin improved food intake in tumor-bearing mice. Similar results were obtained with respect to body composition

where there was an increase in total body dry weight as well as whole body fat weight in both ghrelin groups of freely fed non-tumor mice but only in high-dose ghrelin group of tumor-bearing mice. There was no evidence of tumor progression in this study.

In a rat sarcoma model, DeBoer et al. [76] injected ghrelin and a synthetic ghrelin mimetic BIM-28131 through continuous infusion using subcutaneous osmotic minipumps. They measured the effect of these compounds on food intake and body composition parameters including lean body mass. They found that administration of ghrelin and this ghrelin mimetic resulted in an increase in food intake as well as in body weight in both groups. However, these effects were only observed at continuous high-dose administration of these agents and were not noted at low-dose or twice-daily subcutaneous injections of ghrelin. There was also decreased loss of body mass in high-dose ghrelin and BIM-28131 when compared to control group. Rats treated with BIM-28131 at both high and low doses lost less fat mass than controls. However, that was not the case with ghrelin and there was no difference in fat mass in rats receiving ghrelin when compared to placebo. Rats on high-dose ghrelin and BIM-28131 maintained lean body mass, which was not observed with low doses of ghrelin and BIM-28131. Ghrelin treatment also resulted in increased hypothalamic expression of orexigenic peptides: agouti gene-related peptide (AgRP) and neuropeptideY (NPY) as well as decrease in expression of the type 1 IL-1 receptor. Like all other animal studies there was no evidence of progression of cancer with ghrelin or BIM-28131.

Human Data Supporting Ghrelin Use in Cancer Cachexia

It has been demonstrated that ghrelin increases food intake in healthy volunteers. However, there are scant data in subjects with negative energy balance, including patients with cancer and anorexia. To date, only a few pilot reports of synthetic ghrelin or ghrelin agonist therapy in cancer cachexia patients have been published.

Neary et al. [77] performed a randomized, placebo-controlled, crossover clinical trial in 7 cancer patients with impaired appetite to determine whether ghrelin stimulates appetite in cancer patients. The main outcome measures were energy intake from a buffet meal after administration of a single dose of synthetic human ghrelin or placebo and meal appreciation as assessed by a visual analog scale. A significant increase in the consumption of calories ($31 \pm 7\%$; $P = 0.005$) was observed with ghrelin infusion compared with saline control, and every patient ate more on his/her ghrelin administration day. The meal appreciation score was greater by $28 \pm 8\%$ ($P = 0.02$) with ghrelin treatment. There was no evidence of a compensatory decrease in food intake after ghrelin treatment as assessed by 24-h food diary. No side effects were observed during the study period. This study does show some short-term benefits of ghrelin in cancer cachexia patient, but whether this will translate into long-term weight gain on in this patient population is not known.

Strasser et al. reported a randomized, placebo-controlled, double-blind, crossover study looking into the safety, tolerability, and pharmacokinetics of intravenous

ghrelin in patients with advanced cancer and anorexia/cachexia [78]. Twenty-one adult patients were randomized to receive ghrelin on days 1 and 8 and placebo on days 4 and 11 or vice versa, which was given intravenously over a 60-min period before lunch. Ten patients received lower dose ghrelin (2 mcg/kg) and eleven received higher doses of ghrelin (8 mcg/kg). These doses of ghrelin were safe and well tolerated by patients and no adverse events were observed. Ad libitum food intake tended to improve during ghrelin administration but this was not statistically significant. Several patients preferred ghrelin to placebo.

Most recently Lundholm et al. published data on the effects of ghrelin administration in patients with solid gastrointestinal malignancy [79]. In this phase 2, randomized, double-blind trial, patients with GI tumors were randomized to receive either daily subcutaneous injections of high-dose ghrelin treatment (13 mcg/kg daily, 17 patients) or low-dose ghrelin treatment (0.7 mcg/kg daily, 14 patients) for 8 weeks. Food intake and energy expenditure did not differ significantly between patients in the high-dose or low-dose group. However, appetite which was scored on a visual analog scale increased significantly in patients receiving high-dose ghrelin. Other findings from this study included decreased basal growth hormone levels after 8 weeks of treatment and statistically significant less pronounced whole-body fat loss in cancer patients who were receiving daily high-dose ghrelin compared with low-dose treatment. Interestingly, there were no statistically significant differences in IGF-1 and ghrelin in the two groups. There were also no adverse events observed in patients on high-dose ghrelin, neither was there any evidence of elevated tumor markers such as cancer antigen 125 (CA 125), carcinoembryonic antigen (CEA), and CA 19-9 suggesting tumor progression.

Human Data Supporting Ghrelin Use in Cancer Cachexia

Human Data Supporting the Use of Ghrelin Mimetics in Cancer Cachexia

Because ghrelin is a peptide with a half-life of 30 min, its efficacy in humans is limited unless it is administered parenterally as a continuous infusion. Ghrelin mimetics are non-peptidic, orally available, small molecules that have a long half-life allowing for once-a-day administration. These compounds have been in clinical trials for over a decade and several studies have demonstrated their safety and efficacy [48, 80, 81].

Anamorelin (formerly known as RC-1291) is the most studied orally available growth hormone secretagogue and ghrelin mimetic in this setting. In a phase 1 randomized, double-blind, placebo-controlled, dose-escalation study of healthy volunteers on anamorelin, subjects who received this drug had significant dose-related weight gain after 6 days when compared to patients who received placebo [48].

Anamorelin also significantly increased blood GH concentrations as well as IGF-1 and IGF-binding protein 3 (IGFBP-3) concentrations at all doses when compared with placebo after 5–6 days of treatment. These significant dose-related increases in body weight directly correlated with changes in IGF-1 concentrations. Anamorelin was well tolerated and there was no dose-limiting side effect reported [46]. To date there has been only one trial that looked into the effects of anamorelin on cancer cachexia. In an unpublished, phase 2, double-blind trial in cachectic patients with multiple cancer types, oral anamorelin (50 mg/day for 12 weeks) significantly increased mean lean body mass and total body mass when compared to placebo. These changes were evident at 4 weeks and maintained for the entire duration of the study (12 weeks). This study also showed improved handgrip strength in patients on anamorelin as well as statistically significant increase of anabolic markers like IGF-1 and IGFBP-3. Moreover, anamorelin was well tolerated. Table 12.1 summarizes the data on the use of ghrelin or ghrelin mimetics in the setting of cancer.

Safety of Ghrelin and Ghrelin Mimetics in Cancer

The long-term safety of ghrelin and ghrelin mimetics is a central issue in the setting of cancer and it has not yet been fully established. A brief rise in GH is observed with ghrelin infusion, causing the theoretical concern of increased risk of cancer development or progression and cell growth in patients on ghrelin treatment given the reported role of IGF-1 in tumor progression and more recently the use of IGF-1 antagonists as anticancer therapies. Also, the GHS-R is expressed in many normal and tumoral tissues including breast, thyroid, and prostate. However, data about the effect of ghrelin on cell lines is conflicting. Some studies show proliferation of certain thyroid tumor cell lines but growth inhibition in others [82, 83]. Ghrelin administration also was shown to inhibit the growth of some breast carcinoma cell lines [84] but to increase growth in some prostate cancer and pancreatic adenocarcinoma cell lines [85, 86].

Nevertheless, when whole animal tumor growth studies have been conducted to evaluate the effect of upregulating the growth hormone/IGF-1 axis (either through growth hormone or ghrelin mimetic administration) the results have generally indicated a beneficial effect [87–89]. There is evidence implicating IGF-1 in oncogenic potential; in addition, there have been reports indicating the therapeutic potential of the major binding protein, IGFBP-3, in attenuating oncogenic behavior either via its ability to bind IGF-1 or via direct, IGF-independent actions. Consequently, it may be that the balanced effect of a growth hormone-based intervention to increase both IGF-1 and IGFBP-3 may be responsible, in part, for some of the apparent discrepancies. As mentioned above several studies have used ghrelin or its mimetics in the setting of cancer in both humans and animals without any evidence to this date that these interventions would increase tumor progression.

Table 12.1 Summary of ghrelin and ghrelin mimetic trials in humans and animals

References	Underlying cancer or cancer model	Species	Agent	Effects
Hanada et al. [68]	SEKI human melanoma cells	Mice	Ghrelin	Weight: Ghrelin 0.2 g weight gain; Saline 0.7 g weight loss Food intake: Increase in food intake in mice receiving high-dose ghrelin
Wang et al. [75]	Methyl-cholanthrene-induced sarcoma	Mice	Ghrelin	Weight: Increase in total body and fat weight in high-dose ghrelin-receiving animals Food intake: Increase in food intake in mice receiving high-dose ghrelin
DeBoer et al. [76]	Methyl-cholanthrene-induced sarcoma	Rats	Ghrelin, BIM-28131	Weight: 13% gain in ghrelin/tumor group; BIM-28131/tumor group 19.5% gain; control group 10.3% weight loss Food intake: Ghrelin 37% more than saline group; BIM-28131 43% more than saline group Decrease in lean body mass: Ghrelin 1.0%; BIM-28131 2.7%; and saline group 12.6%
Neary et al. [77]	Melanoma, breast, colon cancers	Humans	Ghrelin	31% increase in consumption of calories in ghrelin group
Garcia et al. (unpublished)	Different cancer types	Humans	Anamorelin	Total body mass: Anamorelin +0.6 kg; placebo −1.45 kg Lean body mass: Anamorelin +1.75 kg; placebo +0.4 kg
Strasser et al. [28]	Different cancer types	Humans	Ghrelin	No significant difference in food intake in ghrelin and placebo groups
Lundholm et al. [79]	Multiple GI malignancies	Humans	Ghrelin	Appetite higher in high-dose ghrelin group in comparison to low-dose group Whole body fat loss 10.6% in high-dose group and 23% in low-dose group

Use of Ghrelin in Chemotherapy-Induced Cachexia

Cancer chemotherapy is associated with multiple undesirable side effects including nausea, vomiting, dyspepsia, neuropathy, and cachexia. Most of the gastrointestinal side effects of cancer chemotherapy are related to delayed gastric emptying and gastric motility issues. Given the presence of numerous ghrelin receptors in the GI tract and the fact that ghrelin has prokinetic properties, ghrelin has been postulated as an agent to prevent GI-related side effects related to chemotherapy. Liu et al. [90] reported the role of ghrelin in a rat and mouse model of gastric stasis induced by cisplatin which caused significant reduction in feeding. There was a 16-fold increase in food intake over 1 h in cisplatin/ghrelin-treated rats when compared to cisplatin/vehicle-treated rats. Similar results were reported in mice where daily food intake significantly increased in the cisplatin/ghrelin-treated mice as well as saline/ghrelin-treated mice. In a ferret model of cisplatin-induced emesis and cachexia, Rudd et al. [91] reported no reduction in cisplatin-induced retching and vomiting after intraperitoneal administration of ghrelin. However, intracerebroventricular administration of ghrelin reduced the number of retches by 61% and at a higher dose intracerebroventricular ghrelin reduced the number of episodes of both vomiting as well as individual retches.

Besides causing GI side effects, cisplatin is also notorious for causing cachexia irrespective of the presence or absence of cancer. Our group in the past studied the role of ghrelin in cisplatin-induced cachexia and neuropathy in a rat model [92]. Animal subjects were given cisplatin, ghrelin, ghrelin–cisplatin, or vehicle intraperitoneally. Animals in the cisplatin group lost weight in comparison to the cisplatin/ghrelin group, which actually gained weight. Moreover, animals in the cisplatin group had a significant decrease in food intake while they were on cisplatin; on the other hand administration of ghrelin with cisplatin totally normalized food intake. Lastly, ghrelin prevented the decrease in IGF-1 levels induced by cisplatin but had no effect on plasma IGF-I levels in control rats.

Conclusion

So far ghrelin's data in cancer cachexia patients, though scant, are promising. Increased appetite, prevention of loss of fat mass, and improvement in lean body mass suggest that ghrelin may certainly help patients suffering from cachexia syndrome due to cancer and other chronic diseases. However, use of ghrelin will be limited as it can only be administered parenterally and it has a very short half-life. However, ghrelin mimetics can overcome this limitation and are currently under clinical development. More studies are needed looking into the long-term safety and efficacy of ghrelin and its mimetics in cancer patients and other chronic diseases associated with cachexia.

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Chapter 13
Ghrelin in Cachexia Associated with End-Stage Renal Disease

Damien Ashby, Peter Choi, and Stephen Bloom

Abstract Much of the high mortality seen in chronic renal failure is due to a form of vascular disease which, unlike the overnutrition vascular disease of the general population, is closely associated with the development of malnutrition. Various micronutrient deficiencies occur, and are commonly treatment related, but a specific syndrome of energy malnutrition associated with unexplained activation of inflammatory mediators, is a common and early feature of renal failure, which is frequently refractory to nutritional supplementation. Many abnormalities of appetite hormones have been found in renal failure, and like obesity, renal malnutrition is increasingly understood as a disorder of appetite homeostasis, which therefore would respond best to manipulation of the appetite regulatory system—for example by administration of ghrelin. Reduced clearance by failing kidneys leads to accumulation of ghrelin, with elevated circulating levels of total ghrelin; however, a reduction in the acyl fraction has been found. Acyl ghrelin administration has been shown to increase energy intake in animal models of renal failure, and promising results have also been found with daily subcutaneous injections in dialysis patients over the course of a week. In addition, ghrelin is thought to have an anti-inflammatory effect, which may be therapeutically relevant in a syndrome in which inappropriate inflammation is a prominent feature.

D. Ashby, M.R.C.P., Ph.D. (✉)
Hammersmith Hospital, Imperial College Kidney and Transplant Institute,
Du Cane Road, London W12 0HS, UK
e-mail: d.ashby@imperial.ac.uk

P. Choi, M.A., M.B., B.Chir., Ph.D., F.R.C.P.
Imperial College Healthcare NHS Trust, Hammersmith Hospital, London, UK

S. Bloom, M.D., D.Sc., F.Med.Sci.
Department of Endocrinology, Imperial College Healthcare NHS Trust, London, UK

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Chronic Renal Failure

Renal Replacement Therapy and Mortality

Chronic renal failure is the irreversible impairment of kidney function, of any severity. End-stage renal disease is severe chronic renal failure in which, without renal replacement therapy, survival would be limited to a few months due to inadequate excretory function, which by this time is usually less than 10% of normal. Renal replacement therapy (RRT) refers to dialysis and transplantation: treatments developed in the 1960s which have the potential to sustain life indefinitely when the kidneys are no longer working adequately. These treatments were initially restricted to selected patients, but over the last 30 years have become far more widely available. During 2007, for example, 6,613 new patients started RRT in the UK, so that by the end of 2007, there were 45,484 individuals in the UK on maintenance dialysis or with a functioning kidney transplant [1], representing an increase of 11.8% since the previous year. In many countries there is therefore a large and growing population of people sustained by RRT.

The ideal form of RRT is a kidney transplant both in terms of quality of life and survival, but dialysis is the only option for many patients. This is due in part to a shortage of donor organs and therefore waiting lists for transplantation, in part to the age and comorbidity of this population, which increases the operative risks so that transplantation is considered too hazardous, and in part due to immunological barriers to transplantation, particularly in those requiring repeated transplants. At the end of 2007 in the UK, just over half of the RRT population (24,352, 53.4%) were on some form of dialysis, and only around a third of these were actively listed for transplantation.

Although dialysis is life-sustaining, it does not restore full health, and patients suffer a significant reduction in quality of life, partly from the impact of therapy on patient's lifestyles, and partly from ongoing symptoms and secondary pathologies which dialysis therapy is unable to resolve. More importantly however, mortality in dialysis patients is greatly increased. The one year survival (censored for transplantation) of prevalent dialysis patients according to age is shown in Fig. 13.1. Overall survival adjusted to age 60 is 88.5%, and at every age group is greatly increased relative to the healthy population, with the most striking differences seen in young adults whose risk of death is increased 20-fold [2].

The commonest causes of death amongst dialysis patients are cardiovascular diseases, including myocardial infarction, stroke and sudden cardiac death, which together account for up to half of all deaths. Infections, which sometimes are related to dialysis access devices, constitute the second largest group. In many populations, withdrawal of treatment at the patient's request is also a significant contributor.

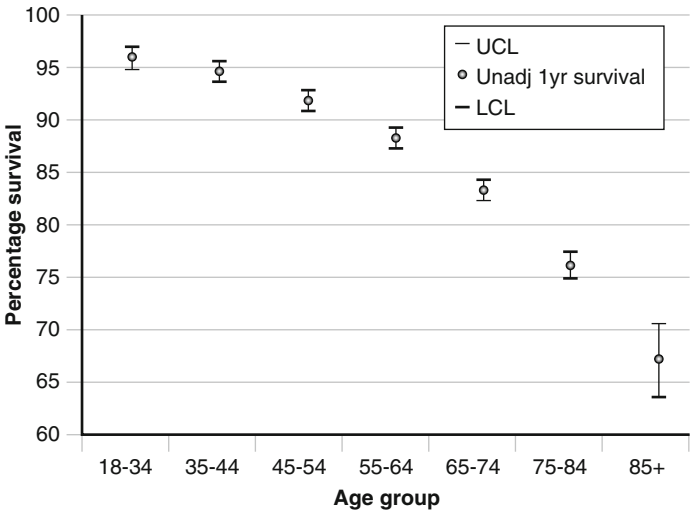

Fig. 13.1 One-year survival of prevalent dialysis patients in the UK at the start of 2007 by age group (Ansell et al. 2009. Reprinted by permission from UK Renal Registry)

Cardiovascular Disease in Chronic Renal Failure

That cardiovascular disease should be the predominant cause of death in dialysis patients seems logical, since the well-known risk factors for cardiovascular events, such as hypertension, hypercholesterolaemia and obesity are common in patients with chronic renal failure. Initially therefore, it was reasoned that outcomes might be improved with enthusiastic treatment of these risk factors: antihypertensives, cholesterol lowering agents, and lifestyle advice focused on weight loss and smoking cessation. With the exception of smoking cessation however, risk factor modification has been largely unsuccessful in improving cardiovascular outcomes in dialysis patients. As an example, in the "4D" study (Die Deutsche Diabetes Dialyze) in 1,200 dialysis patients with diabetes, randomly assigned to cholesterol lowering with atorvastatin versus placebo, no differences in survival or cardiovascular endpoints were observed over a follow up period of 4 years [3].

One reason behind the negative findings in this and other studies may be that the traditional risk factors in the non-renal population are not such potent risk factors in dialysis patients. For example, in a cohort of 1,100 dialysis patients followed for up to 10 years [4], levels of circulating cholesterol at baseline were strongly associated with survival, but contrary to expectation, the best outcomes were seen in the group with the highest baseline cholesterol (Fig. 13.2). Similarly, the relationship between body mass index (BMI) and survival is not as one would expect, so that patients

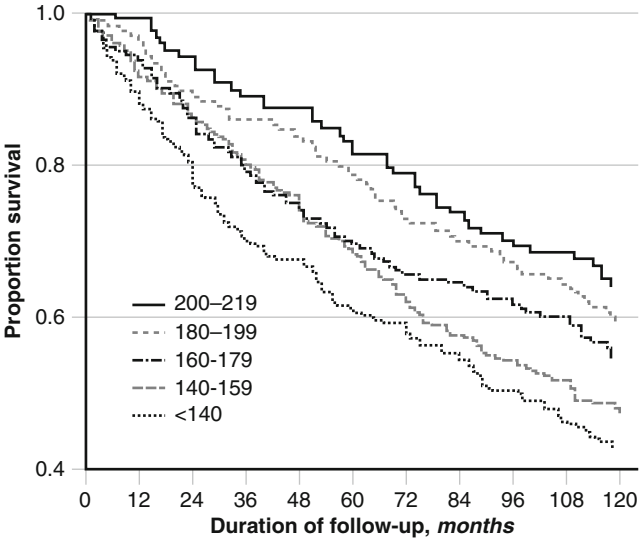

Fig. 13.2 Survival curves over 10 years in a cohort of 1,167 dialysis patients separated according to quintiles of baseline plasma cholesterol (Iseki et al. 2002. Reprinted by permission from Macmillan Publishers Ltd)

with the highest BMI have the best outcomes [5]. The striking reversal of the usual relationships between traditional cardiovascular risk factors and mortality is often termed "reverse epidemiology" [6].

Consistent with a different risk factor relationship, the dominant cardiovascular pathology in dialysis patients is different from that in the general population. In terms of coronary pathology, for example, diffuse calcification detected by CT scanning, is particularly common [7], is related to the degree of renal impairment, and often occurs in the absence of focal atheroma, although traditional atherosclerosis may occur also [8]. Ventricular hypertrophy is also marked, even in the absence of hypertension, being present in over half of normotensive patients not on dialysis [9], and three quarters (75.9% of 108 patients) of patients receiving haemodialysis [10]. In clinical terms differences also exist: in a large series of hospital admissions due to ischaemic cardiac events, patients with renal failure were less likely to present with chest pain. ST segment elevation was also less frequently seen on ECG, but arrhythmias and in-hospital deaths were more common [11].

Also, unlike traditional atheromatous disease in the general population, which is associated with overnutrition, this pathology is associated with undernutrition, which is a common feature of kidney failure, and this perhaps explains this reversal of risk factor effects. For example, in 109 patients not on dialysis, there was substantial overlap between the presence of malnutrition by subjective global assessment (SGA), inflammatory markers and carotid intimal thickness as measured by ultrasound [12]. In patients on peritoneal dialysis, nutritional and inflammatory markers have similarly been found to be associated with coronary calcification

by CT scanning [13]. The frequent coexistence in patients with renal failure of malnutrition raised inflammatory markers and cardiovascular disease is suggestive of a clinical syndrome, with a singular underlying pathophysiology, for which the term "malnutrition inflammation cachexia syndrome" has been coined.

Characteristics of Malnutrition in Chronic Renal Failure

Energy Malnutrition

Disease-related malnutrition refers to the decline in nutritional state which is a common feature of many chronic diseases, including cancer, heart failure, and chronic kidney disease (CKD). It involves the loss of both fat and protein stores, the latter coming largely from skeletal muscle, leading to weight loss and weakness. Although the hallmark of malnutrition is weight loss, this may be difficult to detect in the early stages, or masked by other factors such as fluid retention, but malnutrition can be said to exist when there is a persistent state of negative energy or protein balance. It therefore becomes most apparent as the disease progresses, and is often thought to be a feature of "end stage" disease, but in reality, it has been present since very early in the patient's illness, and may have been amongst the presenting features when the disease was first diagnosed.

Malnutrition has been found to predict high mortality in many chronic illnesses other than renal failure. Weight loss or low BMI are poor prognostic factors in heart failure, [14–19], emphysema [20–22], cirrhosis of the liver [23], and rheumatoid arthritis [24, 25]. Similar findings have been made in many cancers [26–30] with the possible exception of breast cancer, where low BMI is not associated with increased mortality, although the relationship between outcome and hypoalbuminaemia is preserved [31, 32].

The frequent occurrence of malnutrition in renal failure and link between poor nutritional state and mortality was first suggested in studies of dialysis patients during the 1970s [33, 34], and nutritional indices have now been firmly established as prognostic markers in very large studies of 53,933 [35] and 45,917 haemodialysis patients [36]. Malnutrition is common in CKD, being evident in many patients when glomerular filtration rate (GFR) is still over 30 ml/min [37, 38], and whilst the initiation of dialysis is associated with improved nutrition, this is frequently temporary and malnutrition becomes increasingly common in patients who remain on dialysis for a long time [39]. As well as mortality, the rate of intercurrent illness and hospitalisation is linked to nutritional status [5].

Body size and composition are perhaps the most obvious markers of nutritional state. A low BMI was identified as a risk factor for mortality in a study of 45,917 patients followed for 1 year [36]. In this study the relatively short follow-up raises the question of confounding by comorbid illness, but in a similar study the link with mortality was not explained by an interaction with comorbidity [40]. Weight changes

over time and BMI are useful indicators of total body mass, but not all weight is equal. Muscle mass may be preferentially lost as wasting occurs, has more consistently been associated with renal failure [41] and may be more closely associated with poor outcomes.

For example, in a study including data from over 70,000 dialysis patients, Beddhu et al. confirmed that higher BMI is associated with better survival, but by using 24 h urinary creatinine excretion as a marker of muscle mass, demonstrated that the benefit of high BMI was limited to those with higher muscle mass, so that increased fat mass alone conferred no survival benefit [42]. However, a major limitation of this study is the validity of creatinine excretion as a marker of muscle mass. Although often estimated by anthropometric or other methods, the most accurate determination of muscle mass in practice requires cross-sectional imaging, by which not only the quantity but also the distribution can be assessed. The availability of these scans limits this technique to small studies however, so that most studies employ other methods.

Dual-energy X-ray absorptiometry (DEXA), which measures the attenuation of signals from two energy sources to assess tissue composition, is perhaps the most studied technique. Using this method, in terms of prognostic implication, both lean and fat tissue appear to be important: when measured by DEXA in a study of 808 haemodialysis patients, reductions in both lean and fat mass were associated with mortality over a follow up period of 4.5 years [43].

Bioimpedance analysis (BIA), which measures the impedance of an alternating electrical current, is frequently used as a more convenient, non-invasive method of measuring body composition, although limitations may exist in renal patients due to abnormalities in the distribution and accumulation of fluid compartments. However, in a study of 131 haemodialysis patients, Maggiore demonstrated a relationship between body composition measured by BIA and mortality, confirming the utility of this technique [44]. Interestingly, however, the BIA parameter most closely related to outcome was phase angle, which reflects the relative contributions of resistance (which inhibits current in phase with voltage) and reactance (which inhibits current out of phase with voltage, often termed capacitative resistance since a capacitor only allows current to flow in the presence of changing voltage) to overall impedance to an oscillating voltage. However, phase angle is not obviously related to nutritional status, and may reflect some other unknown aspect of tissue integrity.

Biochemical markers of nutrition are amongst the easiest to measure, and several have been associated with mortality in dialysis patients including most consistently albumin, and cholesterol as in a 6-month study of 7,819 dialysis patients [45]. Other biochemical markers have also been linked with outcome including pre-albumin [46], parathyroid hormone [47] and urea [34]. The purity of these biochemical indices as markers of nutrition is questionable, since they are also influenced by other pathophysiological states, such as infection or inflammation. However, they clearly also reflect a very stable patient characteristic, which is highlighted by their association with outcome over many years of follow-up: albumin, pre-albumin and cholesterol continue to be associated with mortality after 10 or 12 years of observation [46, 47].

For purer indicators of nutritional status one can examine energy intake and expenditure. Self-reported appetite was found to be an indicator of mortality in 331 haemodialysis patients followed for 12 months [48], and as a more objective measure of dietary intake, Shinaberger examined protein nitrogen appearance (PNA) in 53,933 patients, finding that low PNA, and in particular falling PNA, were predictive of mortality over 2 years [35].

Resting energy expenditure (REE), measured by indirect calorimetry, has also been investigated in kidney patients. Reduced GFR was associated with lower rather than higher REE in one study of 15 patients with CKD, but this relationship was confounded by the association between both parameters and lean mass [41]. In a study of 55 haemodialysis patients and 55 controls, REE was found to be similar between the groups, but related to inflammatory markers in the dialysis group [49]. In the largest study, in which 251 patients on peritoneal dialysis were followed for 28 months, increased REE was associated with higher mortality and cardiovascular mortality in particular, and was also related to biochemical markers of inflammation and malnutrition such as CRP and albumin [50].

Thus, macronutrient malnutrition is a common and early complication of chronic renal failure with profound prognostic implications. A wide variety of characteristics may be observed and this has led to some confusion regarding terminology and definitions. At a recent expert panel meeting some consensus was achieved regarding the assessment and classification, with "protein-energy wasting" said to be diagnosed by fulfilment of specific criteria in at least three out of four categories: biochemical indices (albumin, pre-albumin or cholesterol), total body mass, muscle mass and dietary intake.

Micronutrients

As well as energy malnutrition, abnormalities of micronutrients are common in CKD, occurring for various reasons, including reduced dietary intake (in some cases due to therapeutic dietary restrictions), increased utilisation, and nutrient losses into the dialysate fluid. Water-soluble vitamins are particularly susceptible to loss into the dialysate fluid [51] and were found to be deficient in the plasma and circulating red blood cells in many patients in the earlier days of dialysis therapy [52], but can be simply restored in most cases with a daily oral supplement [53]. This is clinically important since complications can be attributed to deficiencies—a low level of vitamin C for example is a risk factor for cardiovascular mortality in haemodialysis patients [54], and therapy with vitamin C has been found to reduce resistance to erythropoietin [55, 56].

The situation for trace elements is more complicated since deficiency states are less easy to measure and define. Loss of trace elements into the dialysate fluid is much less of a problem because of avid protein binding in the circulation, and toxicities from dialysate contaminants such as lead or aluminium are more common than deficiencies.

Plasma zinc, for example, is frequently low in dialysis patients, although levels in circulating leucocytes are normal [57, 58]. Supplementation of zinc has been reported to result in improvements in impotence [59, 60] and neuropathy and taste sensation [61], and may also have a role in improving the response to erythropoietin [62]. In one randomised controlled trial of oral zinc supplementation in haemodialysis patients defined as zinc deficient on the basis of plasma levels, 6 weeks of treatment resulted in improved zinc levels and a trend towards a reduction in CRP, from 13.5 to 10.5 mg/l [63].

The term conditionally essential may be applied to an amino acid, such as cysteine, which is essential when dietary methionine (the other sulphur containing amino acid) is low, but not essential if sufficient methionine is available. However, it could be argued that the unusual situation of haemodialysis makes other nutrients essential that would not normally be so. The amino acid coniine, which is involved in fatty acid transport, may well fall into this category.

No clinical syndrome of nutritional carnitine deficiency exists: it can be synthesised from lysine and methionine. In the past some brands of formula milk did not contain carnitine, but infants fed exclusively on these formulas grew and developed normally, although some minor differences in plasma free fatty acids were reported [64]. However, a mutation of the carnitine transporter results in systemic carnitine deficiency which is characterised clinically by myopathy, hypoglycaemia and hyperammonaemia, and is fatal if untreated [65], and less profound carnitine depletion occurs in several other settings possibly associated with myalgias and other symptoms. Carnitine is lost into the dialysate fluid, and low plasma and muscle levels have been found in dialysis patients [66], with rapid reductions occurring over the first year on haemodialsis treatment [67]. Supplementation has been suggested to improve lipid profiles [68], and there is some evidence of a role in anaemia. In vitro, carnitine reduces red cell osmotic fragility and enhances bone marrow cultures [69, 70], whereas clinically, low levels have been associated with erythropoietin resistance [71], and a systematic review concluded that supplementation improves erythropoietin sensitivity in haemodialysis patients [72].

Thus, as well as macronutrient (protein and energy) deficiency, a variety of micronutrient abnormalities is common in chronic renal failure, contributing to symptoms, secondary pathologies and reduced survival (Table 13.1).

Energy Homeostasis in Chronic Renal Failure

Mechanisms of Energy Malnutrition

For micronutrients, losses into the dialysate fluid create an increased requirement, which leads to deficiency if the increased requirement is not met from the diet, but there is very little calorie loss into the dialysate fluid, so energy malnutrition must result from either increased energy expenditure or reduced energy intake.

Table 13.1 Micronutrients in chronic renal failure[a]

Water-soluble vitamins with higher than usual dietary requirements in dialysis patients due to abnormal losses into dialysate	B	Erythropoietin resistance
	C	Erythropoietin resistance
		Cardiovascular disease
		Oxalate toxicity with replacement
Active vitamin D required due to impaired renal metabolism, but nutritional deficiency is also common	D	Osteodystrophy
		Myopathy
Fat soluble vitamins with no tendency to deficiency in chronic kidney disease	A	Bone toxicity with replacement
	E	Neuropathy
	K	Bleeding
Abundant minerals which are renally eliminated, generally requiring dietary restriction to maintain homeostasis	Na	Hypertension
		Oedema
	K	Arrhythmia
	PO_4	Vascular calcification
		Hyperparathyroidism
	Mg	Myopathy
		Diarrhoea
Essential trace minerals with complex disorders of malabsorption and abnormal losses, usually requiring supplementation	Zn	Neuropathy
		Impotence
	Fe	Erythropoietin resistance
Toxic water based trace minerals which must be removed from dialysate to prevent accumulation in haemodialysis patients	Al	Dementia
		Adynamic bone disease
		Myopathy
	Pb	Dementia
		Erythropoietin resistance

[a]Summary table showing groups of micronutrients with possible deficiencies or toxicities in chronic renal failure

The data on energy expenditure are not completely in agreement, but overall there appears to be no substantial difference between dialysis patients and healthy controls when allowing for differences in lean body mass [41, 49]. The major contributor to negative energy balance is therefore believed to be poor energy intake due to reduced appetite, i.e. uraemic anorexia [48].

Occasionally in renal patients this anorexia is due to a clear secondary complication which is known to influence appetite such as gastritis, a concurrent infection, or inadequate dialysis, but for the most part the reduction in appetite occurs when no reversible factor can be identified. Malnutrition is persistent in these cases after thorough search for and treatment of these factors [73].

One secondary pathology which deserves consideration as a cause of malnutrition is inflammation. As is the case for a number of chronic illnesses related to failure of other organs, CKD is increasingly recognised as an proinflammatory condition, characterised by increased circulating levels of inflammatory mediators in a larger than expected proportion of patients. A strong relationship has been observed between inflammation and malnutrition in this setting, and these have been linked in addition with vascular calcification [12] perhaps explaining the relationship with

mortality. This association has led some investigators to the conclusion that cachexia is caused by inflammation, that the driver of this process is the inflammatory state, caused by uraemic toxins and by contact with the extracorporeal dialysis circuit. Indeed, several cytokines associated with this inflammation, such as IL-1b and TNFa, are known to reduce appetite [74, 75] accounting for the negative energy balance, and the most powerful nutritional predictors of mortality, such as albumin, are also influenced by inflammation [45, 47].

However, the predominant direction of this association is far from clear: as is best known for leptin, there is increasing evidence to suggest that appetite regulatory factors often have inflammatory or cardiovascular effects [76]. Neither the inflammation nor the malnutrition mechanism is well understood, and it would be more logical to say simply that they have a shared pathogenesis, so that dysregulation of the same system simultaneously causes malnutrition as well as the inflammation. In other words, there is a syndrome of malnutrition and inflammation which is associated with cardiovascular disease and mortality in advanced CKD.

It is worth reiterating that the predictive ability of some nutritional parameters has been demonstrated in studies with 10 or 12 years of follow up data [46, 47]. The fact that nutritional indices predict mortality far in advance, independent of and better than other prognostic variables, suggests that cachexia in itself has a detrimental effect on survival. It is therefore not merely a symptom, with no significance beyond being unpleasant for patients, but a complication, with its own adverse outcomes, and a legitimate object of life-prolonging (as opposed to palliative) treatment. Although obvious, this issue is important, since it is frequently doubted or overlooked by clinicians, and because it implies that any debate about the direction of the relationship between inflammation and malnutrition is irrelevant. It is the wrong question—the correct question is whether a sustained improvement in nutritional state leads to a survival benefit. This can only be addressed with interventional studies, but for the most part remains unanswered due to the difficulty in achieving a sustained nutritional improvement.

A few studies of nutritional supplementation have demonstrated an improvement in serum albumin, but there is no convincing data on clinical outcome, and one striking feature of many studies is the high rate of dropout due to non-compliance, as in one study of 85 haemodialysis patients given a nutritional supplement during dialysis for 6 months, in which albumin increased by around 3 g/L in those completing the study, but only 39 did complete the study [77]. To understand the poor compliance in these studies requires a deeper understanding of appetite homeostasis than we have so far considered.

Malnutrition therefore appears to be a primary complication of renal failure itself, for which the pathogenesis is unclear. The question of whether an improvement in nutritional state would lead to improved survival is an important one, but remains unanswered, at least in part due to the surprising difficulty in achieving a sustained nutritional improvement. In order to understand this, we need to focus on appetite, and how this is normally regulated—because, despite the influence of numerous external factors, appetite is surprisingly tightly controlled.

Dysregulation of Appetite in Chronic Renal Failure

Before discussing how appetite is regulated it is worth considering why it should be regulated at all. The fact that we have the desire to eat is such a fundamental part of our existence, and it is sometimes difficult to appreciate that it is a subconscious desire which is physiologically regulated in the interests of energy homeostasis.

In the short term, appetite depends on an enormous number of factors, which doubtless include the body's need for energy, but more frequently are dominated by other considerations: it appears to be a choice, dependent on palatability of the available food, social context and personality. However, the long-term picture is quite different, and this can be appreciated with a simple calculation. Although body weight tends to increase slightly during much of our adult lives, it is remarkable for its stability, with a yearly weight change of less than 1 kg in most of the population [78]. The excess energy needed to induce this weight gain has been variably estimated, but is in the region of 30 MJ [79] compared to an average yearly energy intake of over 3,000 MJ. This means that most of us manage to match energy intake to equal energy expenditure, with an error of less than 1%. It was long suspected therefore, that a regulatory system existed to control appetite in order to maintain energy homeostasis [80], and this theory was confirmed by the subsequent discovery of some of the elements of this system, beginning with the discovery of the adipose tissue hormone leptin, which gives feedback to the brain about the adequacy of the reserves of energy stored in the form of fat.

Thus, a homeostatic system exists for the regulation of appetite: obesity is now firmly accepted to be a disorder of this homeostatic system, and it is likely that malnutrition in chronic renal failure also represents a disorder of this homeostatic system. This means that processes in which significant changes in appetite and nutritional state occur must involve the mediators of this system, and importantly, that interventions to improve nutritional state are unlikely to be successful if they do not affect, at least indirectly, the mediators of this system.

Understanding cachexia as a disorder of a homeostasis explains the difficulty encountered in increasing long-term energy intake in clinical trials, which is seen even when calorie supplements are administered parenterally. For example, in a randomised trial of intradialytic parenteral nutrition in haemodialysis patients, both groups received an oral supplement, but the intervention group also received an intravenous supplement of around 25 kJ/kg at each dialysis session (3 times per week) for 12 months [81]. There were modest improvements in albumin and body weight in both groups, but no benefit in clinical outcomes, and no differences between the 2 groups. One striking finding was that spontaneous energy intake (i.e. what the patients were eating at home, in between dialysis sessions) gradually declined over the first 6 months of the trial in both groups, and even more so in the intervention group, so that the total energy intake was little changed in either group during most of the trial. To compensate for the supplements being taken, appetite for voluntary intake was gradually being reduced by this homeostatic system, restoring the total energy intake to its previous inadequate level.

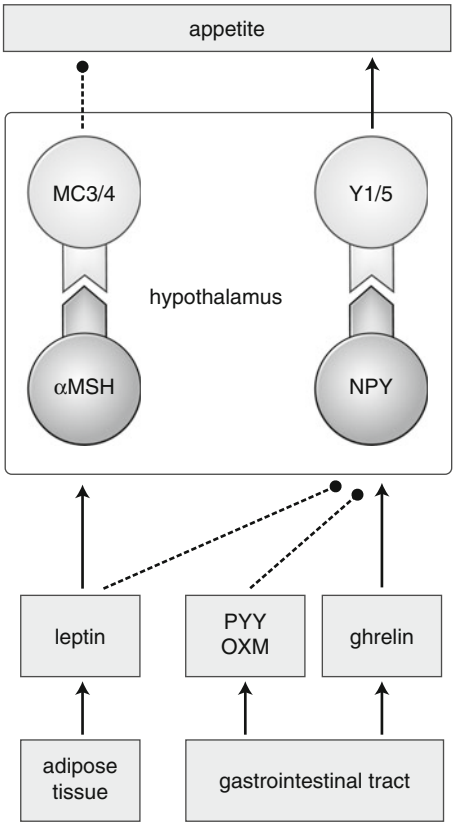

Fig. 13.3 Simplified model of homeostatic appetite control. Appetite is governed by the opposing actions of 2 populations of neurons within the hypothalamus: those releasing melanocortin stimulating hormone (αMSH) which act via the MC3 and MC4 receptors and reduce appetite, and those releasing neuropeptide Y (NPY) which act via the Y1 and Y5 receptors to increase appetite. Several circulating hormones affect appetite largely through their action on these hypothalamic neurones, including leptin, ghrelin, and a number of "satiety" hormones which are released after meals—these include peptide YY (PYY) and oxyntomodulin (OXM). Elevated levels of many of these circulating mediators are seen in chronic renal failure

We have thus far reasoned on theoretical grounds, that cachexia is likely to be due to some change in appetite regulation, but have not considered any direct evidence. However, different lines of evidence are beginning to emerge that firmly implicate appetite control in the pathophysiology of cachexia.

Firstly, a number human observational studies have found that circulating negative regulators of appetite are elevated in patients with chronic illnesses and cachexia. Most attention has focused on leptin, which is higher than expected in heart failure [82, 83] as well as in CKD [84, 85] although other hormones are also involved, such as PYY, which is also elevated in these conditions [86]. A simplified model of appetite regulation, showing the hypothalamic circuit and some its circulating influences, is presented in Fig. 13.3: most of these circulating factors are found at abnormal levels in renal failure.

Leptin is perhaps the best studied in renal disease. In healthy individuals, circulating leptin level reflects total fat mass, and is thus a marker of overnutrition. Although elevated in chronic renal failure, the positive relationship between plasma leptin and fat mass has been found to be preserved by most investigators—thus,

although leptin may be elevated in these conditions, a correlation between the degree of elevation and the severity of malnutrition is not seen, at least in part because the starting position is the opposite relationship with nutritional state. However, where the leptin–fat mass ratio has been calculated, it has consistently been found to be higher in renal patients than controls [87, 88] suggesting a poorer nutritional state than would be expected at high leptin levels. Cross-sectional studies have found leptin to be correlated negatively with calorie intake [89] and lean mass [88], but the most persuasive evidence comes from longitudinal studies in which higher levels are associated with nutritional decline. In 36 peritoneal dialysis patients weight loss over 1 year was correlated with a rise in leptin [90], and in 57 haemodialysis patients baseline leptin was strongly associated with subsequent weight loss over the next 17 months, thus strengthening the likelihood that this association is indeed causal [91].

A second line of evidence implicating appetite control pathways in the pathogenesis of uraemic malnutrition comes from animal studies which have shown prevention or reversal of cachexia by deletion or blockade of specific appetite pathways. For example, the cachexia which follows the induction of kidney failure or cancer is much reduced in mice with a targeted deletion in the melanocortin 4 receptor gene, who have an unopposed orexigenic NPY axis [92]. In addition, intraventricular injection of a melanocortin antagonist, which blocks hypothalamic melanocortin signalling, reverses the weight loss in cancer bearing mice [93], and loss of appetite and lean body mass were prevented by peripheral injection of melanocortin 4 receptor antagonists [94, 95].

Although much of the literature concerns tumour related cachexia, similar data have emerged recently for uraemic malnutrition. The induction of kidney failure by subtotal nephrectomy (surgical resection of 5/6 of the total renal cortex), which causes loss of both lean and fat mass in normal mice, induces very little change in body weight in leptin receptor deficient mice [96]—they appear therefore to be almost completely resistant to the nutritional decline which usually accompanies subtotal nephrectomy. It is therefore clear that elements of the hypothalamic appetite regulatory circuit are involved in mediating the malnutrition which accompanies chronic renal failure.

Thus, central appetite circuits mediate malnutrition in kidney disease, and it is known that these circuits usually respond to a number of circulating hormones, the majority of which are anorexigenic. It is also known that at least some of these anorexigens circulate at elevated levels when renal clearance is impaired, and that levels of these mediators may predict nutritional outcomes to some extent. What is missing is direct evidence that uraemic plasma has anorexigenic activity compared to normal plasma—a gap that has been filled in part by plasma fraction studies from the Karolinska Institute. Briefly, plasma ultrafiltrate was collected from patients beginning dialysis treatment, and ultrafiltered in vitro to achieve a fraction with concentration of molecules larger than 500D, with control samples prepared in a similar way from healthy volunteers. Anorexigenic activity was assessed by injection into rats which were subsequently fed by a tube supplying a sucrose solution continuously into the mouth, with time to food rejection being measured.

The uraemic plasma fractions exhibited marked anorexigenic activity, with earlier rejection of the sucrose solution, indicating directly that compared to control plasma, uraemic plasma contains appetite inhibitory factors, and that these factors are larger than 500D in size.

It seems therefore that renal impairment leads to elevated levels of a number of factors which are sensed by the hypothalamus leading to an adjustment of energy homeostasis in favour of negative energy balance. Cachexia therefore appears to be due to an adaptation of appetite to the diseased state. But, if reduced appetite in these conditions has such adverse consequences, then why should such an adaptive mechanism exist? Although speculative, the answer is perhaps obvious when we consider that short-term illnesses are a more powerful evolutionary pressure than modern chronic diseases, which occur largely in later life. During a short-term illnesses, such as an infection or injury, it would be advantageous for appetite to be diminished, so that food seeking behaviours, like hunting, which would be more hazardous than usual, are postponed until after recovery from the illness. Reduced appetite and cachexia may well be consequences of an adaptive mechanism which is useful in acute illness, but of no advantage in the modern setting of chronic illnesses.

Ghrelin in Chronic Renal Failure

As a circulating mediator involved in the physiological regulation of appetite, the question arises in renal malnutrition as to whether either abnormal ghrelin signalling might be involved in the pathogenesis, or whether the ghrelin pathway might be manipulated for therapeutic benefit.

Observations

Ghrelin is secreted with an acyl side chain on the third serine amino acid which is essential for its neuroendocrine action, and which is rapidly removed in the plasma. After removal of the acyl side chain, desacyl ghrelin is filtered and eliminated by the kidneys. Increasing plasma levels of total ghrelin have been reported with worsening renal impairment [97]. Plasma ghrelin levels are on average doubled compared to controls in patients with end stage renal failure [98], but as in healthy controls, levels are negatively correlated with nutritional indices such as appetite, BMI and albumin [99]. Thus, despite the frequent presence of malnutrition, renal failure is associated with elevated rather than depressed ghrelin levels: a state of ghrelin resistance has therefore been suggested to exist in malnourished renal patients [100]. However, when acyl ghrelin rather than total ghrelin has been measured, normal or low levels have been found in renal failure [101]. Thus, only desacyl ghrelin is elevated - this could be another factor contributing to anorexia as some studies have reported an appetite inhibitory effect with desacyl ghrelin [102].

Therapeutic Potential in Malnutrition

Regardless of whether abnormalities of ghrelin signalling contribute to the pathogenesis of malnutrition in renal failure, the fact that ghrelin is the only established circulating physiological mediator of increased appetite makes it an obvious candidate for therapeutic use. A relatively small but growing literature addresses the use of ghrelin in animal models of renal failure and human disease.

In animal models of disease induced cachexia, ghrelin has been shown to increase food intake and prevent the loss of body weight. This has been seen in rats with wasting induced by repeated lipopolysaccharide injections [103], rats with cardiac cachexia induced by coronary artery ligation [104], and in rats and mice with tumours [105, 106].

In a rat model of chronic renal failure induced by 5/6 nephrectomy, De Boer investigated the effects of continuous subcutaneous ghrelin infusion for 2 weeks, via surgically implanted osmotic pumps [107]. An increase in body weight was observed, which was predominantly due to lean rather than fat tissue, and there was an improvement in the inflammatory profile, with reduced circulating levels of several proinflammatory cytokines including IL-6, TNFα and IL-1β. In a follow up study, the same group demonstrated that the mitochondrial oxidative capacity of skeletal muscle, normally reduced by nephrectomy, was restored by ghrelin infusion. This effect was, at least in part, independent of food intake effect of ghrelin therapy, since it was seen even in ghrelin treated animals who were pair fed with the nephrectomy/placebo group [108].

A few studies have examined the potential for ghrelin to improve nutritional status in human cachexia. Increased appetite has been demonstrated in metastatic cancer patients with anorexia and weight loss, in a randomised crossover study of a single dose of ghrelin given by infusion [109]. On separate days, patients unaware of their treatment allocation, were given ghrelin or saline by infusion after an overnight fast, and then served an excessive quantity of a preselected meal. All 7 patients studied increased their energy intake following ghrelin infusion, by an average of 31%, and reported greater appreciation of the meal.

The longer term anabolic effects of ghrelin have also been demonstrated in non-randomised studies, with twice daily infusions. In 10 heart failure patients, left ventricular ejection fraction, exercise capacity and lean body mass were all shown to increase after 3 weeks of treatment, whilst no change was observed in a control group of heart failure patients hospitalised for diagnostic testing for their cardiac conditions [110]. The same investigators observed similar results in 7 patients with chronic obstructive pulmonary disease, in whom respiratory muscle strength, lean body mass and functional capacity were improved after a 3 week period of treatment [111].

Ours is the only group so far to have investigated the effect of ghrelin in dialysis dependent renal failure. In the first study conducted on 9 patients treated by peritoneal dialysis, a doubling of energy intake immediately following subcutaneous administration of ghrelin was demonstrated, and a 27% increase over the first 24 h period [112].

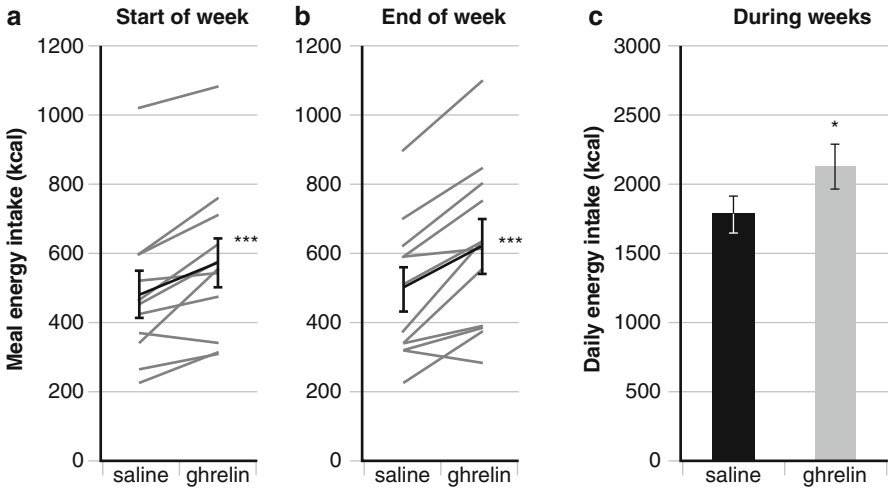

Fig. 13.4 Energy intake in randomised crossover study of daily subcutaneous ghrelin for 1 week in dialysis patients. (**a**) Study meals at the start of saline and ghrelin weeks showing an increase in energy intake after the first injection (individual values and mean+/−se, $p<0.001$); (**b**) study meals at the end of saline and ghrelin weeks showing persistence of the increase in energy intake ($p<0.001$); and (**c**) food diaries during both weeks demonstrating a consistent effect throughout the diurnal period (mean+/−se, $p=0.040$)

Importantly, food intake over the next 48 h period appeared to be unchanged, demonstrating the lack of a compensatory reduction in food intake following the acute period after ghrelin injection.

In the second study, 12 patients (9 haemodialysis and 3 peritoneal) received a week of daily subcutaneous ghrelin (12 μg/kg given 1 h before a meal) and a week of saline, in random order with allocation concealed to participants and investigators [113]. Ghrelin administration led to an 80-fold increase in acyl ghrelin levels and a 20.3% increase in energy intake at the first study meal on day 1 ($p<0.0001$). At the day 8 study meals the appetite effects were similar with a 24.6% increase in energy intake compared to placebo ($p<0.0001$), and the persistence of effect throughout the diurnal period (i.e. without compensatory reduction between the once daily injections) was demonstrated with 3-day food diaries (Fig. 13.4). In this study, ghrelin administration, at the same dose, was of similar efficacy to that reported in controls [114].

Activity related energy expenditure, measured throughout each week by Actiheart pulse and motion monitors, was unchanged in this study, suggesting a positive impact of ghrelin on overall energy balance. The energy expenditure associated with activity is important since this component has been shown to be regulated by nutritional state, and to account for much of the variability in individual responses to changes in food intake [115]. Moreover, it has become apparent that a number of appetite regulators, such as the gastro-intestinal anorectic signal oxyntomodulin, are

also involved in regulation of activity [116]. REE was not measured in this study, but is usually a static component in healthy individuals, thought to be unchanged, or minimally lowered by ghrelin [117, 118].

It seems likely therefore that the acute effect of ghrelin on appetite would translate into a medium term improvement in nutritional state, but in addition, ghrelin is thought to possess a number of other actions which are not obviously nutritional and which may prove to be relevant to the malnutrition of chronic renal failure.

Ghrelin lowers blood pressure by vasodilation in rodents and man, by an effect which, unlike the hypotensive effect of growth hormone via IGF-I, appears to be independent of nitric oxide [119–121]. This leads to an increase in stroke volume and cardiac output without a significant change in heart rate in healthy volunteers as well as patients with heart failure [122–124], and may underlie some of the benefit seen after 3 weeks in patients with heart failure [110]. In dialysis patients there were modest reductions in blood pressure at the time of administration, but no effect beyond about 2 h—this effect therefore does not appear to be therapeutically relevant. In one interesting study, vascular calcification was been shown to be reduced by ghrelin in a rodent model induced by excessive vitamin D [125]. This is of relevance since vascular calcification commonly accompanies malnutrition.

As is the case for a number of chronic illnesses, CKD is considered to be an inflammatory state, characterised by elevated levels of circulating markers of inflammation. Many of these markers, such as C-reactive protein [126] and IL-6 [127] have been closely linked to clinical outcome. The most obvious potential cause of inflammation in haemodialysis patients is the exposure to the extracorporeal circuit [128], but patients not yet started on dialysis treatment already demonstrate elevations in inflammatory markers [129] so other factors are clearly involved. Renal function greatly influences the clearance of many cytokines from the circulation [130], and their retention in renal failure might be expected to cause inflammation, but in addition to elevations of circulating mediators, hyperactivity of inflammatory cell function has also been observed. Many cell types are said to circulate in a "pre-activated" state, so that cytokine production may be normal much of the time, but enhanced responses to stimulation are seen. For example, ex vivo cytokine production in whole blood (which is a close simulation of the in vivo situation) both spontaneously and in response to lipopolysaccharide stimulation, is doubled in dialysis patients compared to healthy controls [131]. Similar pre-activation states have been described for other cell types including peripheral blood mononuclear cells and monocytes [132].

Thus, although the mechanisms are unclear, CKD induces an inflammatory state characterised by elevated levels of circulating inflammatory markers, and enhanced responses of immune cells to stimulation. This inflammatory state is closely related to malnutrition and ghrelin, as well as its nutritional function, is also thought to possess an anti-inflammatory effect.

It had been known for some years that both ghrelin and the ghrelin receptor are expressed by cells of the human immune system, including B and T lymphocytes and neutrophils [133], but the first convincing evidence of an anti-inflammatory role for ghrelin came from studies by Dixit et al. examining pro-inflammatory cytokine

expression in stimulated monocytes and lymphocytes [134]. In freshly isolated human monocytes, T cells and peripheral blood mononuclear cells stimulated with lipopolysaccharide, anti-CD3 or phytohaemagglutinin respectively, transcription and production of IL-1β, TNFα and IL-6 were reduced by ghrelin, whilst several other cytokines were unaffected, including TGFβ, IFNγ and IL-2. In addition, ghrelin inhibited the cytokine response to stimulation by leptin in these cells, neatly suggesting that as well as having opposing effects on food intake, these mediators also have opposing effects on the immune system. They went on to show that pretreatment with ghrelin reduced circulating levels of these cytokines in mice injected with lipopolysaccharide, and attenuated the anorexia which is seen in this model.

A number of other groups have confirmed the anti-inflammatory effect of ghrelin in various in vivo models of inflammation, including chemically induced colitis [135], pancreatitis [136, 137] or arthritis [138], and acute lung injury induced by sepsis [139]. Circulating inflammatory markers and histological grade were reduced by ghrelin, as was the overall clinical severity, and in the more severe models, mortality.

In dialysis patients, although levels of circulating inflammatory cytokines were elevated as anticipated, no changes were observed as a result of ghrelin administration. These measurements however, would have little power to detect changes in dynamic immune responses however, and should not be taken as evidence against a physiologically relevant effect.

Currently available appetite stimulants influence appetite pathways indirectly, have a slower and less reliable onset of action, and are associated with many unwanted effects. There is no immediate change in appetite with megestrol acetate, for example, but increases in energy intake of around 15% have been reported after some weeks [140], with weight gains of around 3 kg over 3 months [141, 142]. However, a quarter of patients are unresponsive [143, 144], a range of steroid-like side effects can occur, and most of the weight gained is fat rather than lean tissue [145]. Acting more directly, ghrelin appears capable of increasing both lean and fat tissues, as shown in rodent models of cancer [105], and CKD [107]. However, several studies found only increases in adiposity [146], consistent with the direct adipogenic effects which have been demonstrated [147], and enthusiasm should also be balanced by the potential for other metabolic effects, including reduced insulin sensitivity, either dependent or independent of growth hormone release [148].

In dialysis patients few adverse effects were reported, and the majority of patients were unable to guess their ghrelin/placebo allocation. Two patients however transiently experienced an uncomfortably strong feeling of hunger within minutes of ghrelin injection, but were able to continue the study on a reduced dose without symptoms. Glucose and insulin levels also appear unaffected by ghrelin administration, but reassurance from short-term studies should be considered in the light of the modest decrease in insulin sensitivity which was reported in a longer duration study using the oral ghrelin mimetic MK677 [149].

One major limitation of treatments based on natural hormones, is the need for parenteral administration due to the large size of the molecule. It is therefore of interest that a number of small molecule analogues are available which are orally absorbed, and are currently being investigated for therapeutic potential. In addition

to MK677 [149], amorelin has shown efficacy in terms of appetite stimulation and weight gain in phase 1 studies [150, 151].

Thus, ghrelin administration does appear to increase energy intake substantially, in a way that is safe, well tolerated, and without apparent tachyphylaxis. In addition, the combination of appetite along with anti-inflammatory effects makes this pathway particularly attractive since manipulation might counter both manifestations of this shared pathogenesis, thereby producing a greater clinical benefit than would be expected from the improvement in energy intake alone. Studies of longer duration will be necessary to determine the effect on nutritional state, and ultimately, clinically relevant outcomes.

Conclusion

A syndrome of malnutrition, closely associated with low grade inflammation and vascular disease, occurs frequently in chronic renal failure, is not reversed by dialysis therapy, and is responsible for a large part of the high mortality seen. The pathogenesis is incompletely understood, but anorexia due to altered levels of circulating appetite mediators undoubtedly plays a significant role, and makes the condition resistant to long-term treatment with nutritional supplements. Although total ghrelin levels are elevated in chronic renal failure, levels of acyl ghrelin are normal or low. Ghrelin shows some promise as a potential treatment for malnutrition in this setting—subcutaneous administration increases appetite and energy intake in the short term in dialysis patients, and appears to be well to be well tolerated, but the effect on energy intake in the longer term is unknown. The extent to which a long-term increase in energy intake could improve the clinical features and survival of cachexia remains unknown, but the ability to achieve the former may soon be a reality, allowing this important question to be answered.

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Chapter 14
Ghrelin Suppression of Autoimmune Encephalomyelitis

Sachiko Miyake and Takashi Yamamura

Abstract Ghrelin is a gastric hormone first identified in the rat stomach as a mediator of growth hormone (GH) release. The biological effect of ghrelin is mediated by a G protein-coupled receptor called GH secretagogue receptor (GHS-R). Although ghrelin is predominantly secreted from mucosal endocrine cells of the stomach, ghrelin and GHS-R are widely distributed throughout various organs including immune cells and lymphoid tissues. Ghrelin induces increases in peripheral blood lymphocyte numbers as well as increases in thymic cellularity and differentiation, thus augmenting cytotoxic lymphocytes and reducing tumor initiation and subsequent metastases. More recent studies have highlighted the anti-inflammatory functions of ghrelin. For example, we have demonstrated that exogenous ghrelin influences the development of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis. In the C57BL/6 mouse model of EAE in which disease is induced by sensitization to myelin oligodendrocyte glycoprotein 35–55 peptide, we found that injections of ghrelin significantly reduced the clinical severity of EAE. The suppression of EAE was accompanied by a reduction in the mRNA levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the spinal cord cellular infiltrates and microglia from ghrelin-treated mice at the peak of disease, suggesting that ghrelin may act as an anti-inflammatory hormone. Consistent with these findings, ghrelin significantly suppressed the production of proinflammatory cytokines in LPS-stimulated microglia in vitro. These results shed light on a new role for ghrelin in the regulation of inflammation with possible implications for the management of human diseases.

S. Miyake, M.D., Ph.D. (✉) • T. Yamamura, M.D., Ph.D.
Department of Immunology, National Institute of Neuroscience, NCNP,
4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
e-mail: miyake@ncnp.go.jp

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Introduction

Autoimmunity is not forbidden but must be well controlled in order not to damage self in a healthy immune system. The development of autoimmune diseases is influenced by both genetic and environmental factors. Nutrition status is one of the factors modulating immune reactions and malnourished people are more susceptible to pathogens. Some patients with autoimmune diseases such as multiple sclerosis (MS) or rheumatoid arthritis (RA) claim that their symptoms are reduced by fasting, although the evidence is mostly anecdotal. Even though the mechanisms linking nourishment and immunity may not be simple, recent studies have shed light on the ability of regulatory feeding hormones such as ghrelin, leptin, and neuropeptide Y (NPY) to modulate immune responses. Serum leptin is decreased after acute starvation, whereas ghrelin and NPY are increased.

Recent advances in the study of helper T (Th) cell subsets suggest that Th1 cells producing IFN-γ (gamma), IL-2, and TNF-α (alpha) and Th17 cells producing IL-17, IL-21, and IL-22 contribute to the pathogenesis of autoimmunity. In contrast, Th2 cells producing IL-4 and IL-5 and regulatory T cells (Treg) are protective against Th1- or Th17-mediated autoimmune diseases [1, 2]. In the context of immunomodulation, starvation leads to immunosuppression or Th2 bias. Exogenous leptin has been reported to modulate the Th1/Th2 balance toward Th1 [3, 4], which is consistent with a decrease in endogenous leptin upon starvation. The effect of NPY seems more complex as exogenous NPY has been reported to ameliorate disease in autoimmune models by suppressing Th1 cytokines [5], although a study using NPY1 receptor-deficient mice showed that NPY promotes the activation of antigen presenting cells in addition to downregulating the Th1 response thereby enhancing rather than suppressing the immune response in vivo [6]. Interestingly, leptin and NPY are linked to ghrelin, the focus of this book, via an endocrine network, since ghrelin stimulates the release of NPY in the CNS [7] while exhibiting antagonistic effects against leptin [8].

The Effect of Ghrelin on the Immune System

Ghrelin is a gastric hormone first identified in the rat stomach in 1999 as a mediator of growth hormone (GH) release [9]. The biological effect of ghrelin is mediated by a G protein-coupled receptor called GH secretagogue receptor (GHS-R). Although ghrelin is predominantly secreted from mucosal endocrine cells of stomach, ghrelin and GHS-R are widely distributed in various organs including immune cells and lymphoid tissues. Koo et al. showed that ghrelin increases the number of lymphocytes in the peripheral blood and thymus and promotes the differentiation of T cells in the thymus [10]. Furthermore, they demonstrated that administration of ghrelin inhibited tumor initiation and subsequent metastases by increasing cytotoxic lymphocytes. These findings suggested that ghrelin possesses immune-enhancing effects that protect and promote healthful conditions.

More recently, it has been reported that ghrelin has anti-inflammatory effects in pathological conditions. The immunosuppressive effects of ghrelin were first

demonstrated in vitro [11] when ghrelin was shown to inhibit activation-induced proinflammatory cytokine production by human monocytes and T cells. Thereafter, in vivo anti-inflammatory activities of ghrelin were demonstrated using a rat model of LPS-induced endotoxic shock. Ghrelin inhibited the production of proinflammatory cytokines including IL-8 and TNF-α (alpha) in human endothelial cells in vitro and suppressed plasma levels of IL-8, TNF-α (alpha) and MCP-1 in rats after LPS injection [12]. Wu et al. also demonstrated that ghrelin suppressed plasma levels of TNF-α (alpha) and IL-6 in a rat cecal ligation and puncture (CLP) sepsis model through activation of the vagus nerve, although they claimed that ghrelin did not inhibit cytokine production from LPS-stimulated macrophages [13]. More extensive analyses of sepsis models by Chorny et al. [14] demonstrated that ghrelin improved survival in three sepsis models including LPS-induced endotoxemia, *E.coli*-induced lethality, and CLP. In addition to the suppression of inflammatory cytokine production, they showed that inhibition of sepsis by ghrelin is mediated by the reduction of high mobility box 1 protein (HMGB1), particularly when ghrelin treatment was started at a later time point [14].

Protection from inflammation was also demonstrated in animal models of inflammatory diseases such as arthritis and colitis. A ghrelin agonist, growth hormone-releasing peptide-2, was shown to decrease the severity of rat adjuvant arthritis in parallel with a reduction of serum IL-6 levels [15]. Moreover, Chorny reported that ghrelin treatment inhibited the severity of collagen-induced arthritis in association with a reduction of HMGB-1 in serum [14]. An anti-inflammatory effect of ghrelin was also shown using 2,4,6-trinitrobenzene sulfonic acid-induced colitis, an animal model of Crohn's disease [16]. In this model, ghrelin inhibited production of inflammatory cytokines and Th1-related cytokines such as IFN-γ (gamma) and IL-12, suggesting an alteration of the balance of T-cell derived cytokines.

Overall, these studies indicate that the anti-inflammatory effects of ghrelin are predominantly exerted via the suppression of proinflammatory cytokine production. Li et al. demonstrated that ghrelin attenuated TNF-α (alpha)-induced nuclear translocation of NF-κB, which serves as a potential mechanism whereby ghrelin modulates inflammatory responses [12]; this result was confirmed by another group using a macrophage cell line [17]. Interestingly, in addition to the attenuation of inflammatory cytokines, they demonstrated that exogenous administration of ghrelin augmented IL-10 production and p38 MAPK activation, which is known to control the release of IL-10 independent of the NF-κB pathway [17]. The enhancement of IL-10 production by ghrelin has been also demonstrated by other groups [14, 16].

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Autoimmune diseases result from chronic inflammation involving autoimmune reactions of lymphocytes. MS is a chronic inflammatory demyelinating disease of the CNS that exhibits a great variety of symptoms and is the most common

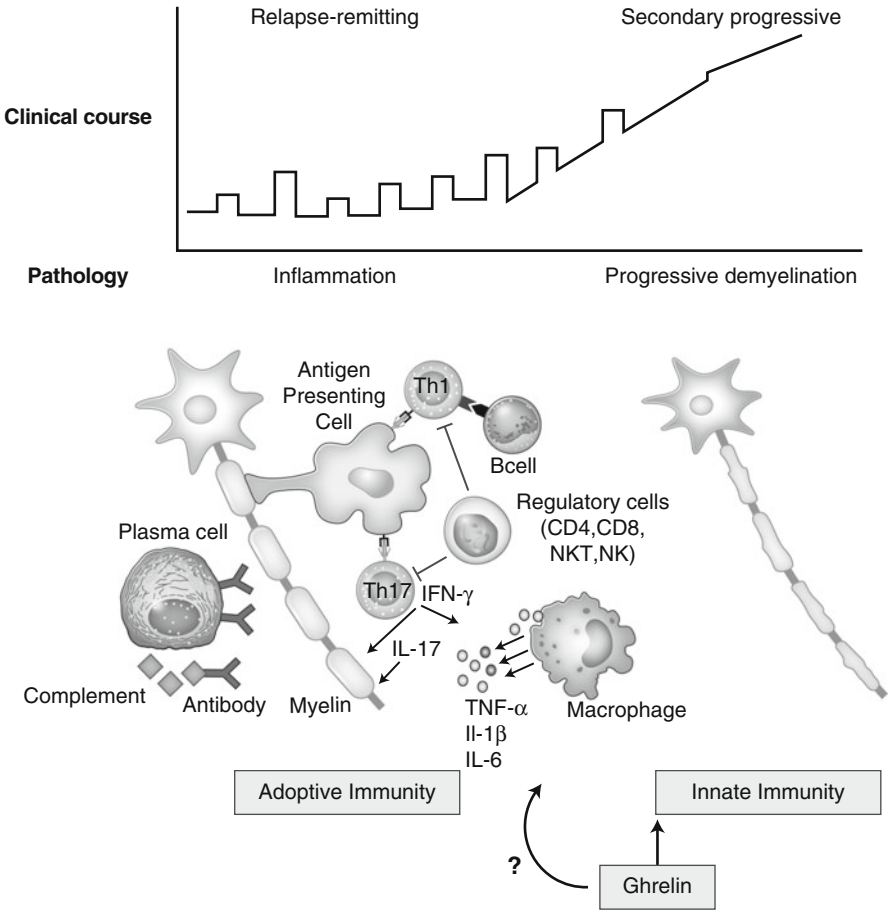

Fig. 14.1 Disease course and immunopathogenesis of multiple sclerosis. The relapsing remitting phase characterized by recurrent attack and recovery is mediated by inflammation caused by adaptive immunity. The secondary progressive phase is characterized by the progressive death of oligodendrocytes and neurons and innate immune activation is speculated. Ghrelin suppresses innate immunity and may also affect adaptive immunity both directly or indirectly

disabling neurological disorder in young adults [18, 19]. Depending upon its clinical course, MS is classified as relapsing remitting (RR), primary progressive (PP) or secondary progressive (SP) (Fig. 14.1). The clinical manifestations of RR patients are characterized by recurrent episodes of acute attack followed by partial or full recovery and about two-thirds of RR patients exhibit progressive neurological disabilities without remission (SP). Some patients develop progressive disease from the onset (PP). Both environmental and genetic factors confer susceptibility to the disease. The pathological features of MS in the CNS are focal areas of demyelination involving infiltration of T cells, B cells, and macrophages within and surrounding the plaques. Even though the primary cause is still unknown, an autoimmune

process against myelin components has been postulated to be the underlying mechanism of inflammation and demyelination [20]. The target antigens include myelin basic protein (MBP), myelin proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG), although the antigen responsible for the pathogenesis has not been clearly proven. Recent results of a clinical trial with altered peptide ligands of MBP support the autoimmune nature of MS. In this trial, administration of altered peptide ligand exacerbated MS in some patients and was accompanied by an increase in Th1 cells reactive to MBP [21]. Moreover, the success of using anti-very late activation-4 antibody treatment to inhibit immune cell entry into the CNS further supports the importance of immune cells in MS [22].

Animal models of CNS demyelination have provided mechanistic insights into the complex pathogenesis of MS. Although there are several models of demyelinating disorders, the most commonly used animal model for MS is an experimental autoimmune encephalomyelitis (EAE) induced in susceptible rodent strains by immunization with CNS antigens such as MBP, PLP or MOG [23]. After immunization, myelin-reactive encephalitogenic T cells, such as IFN-γ-(gamma) producing Th1 cells and IL-17-producing Th17 cells, migrate into the CNS and are reactivated to initiate an inflammatory cascade including the secretion of chemokines that recruit other immune cells such as macrophages and B cells. Proinflammatory cytokines such as TNF-α (alpha), IL-1β (beta), and IL-6 are important for inflammation and tissue damage. The focal plaques of immune cell infiltrates and the following demyelination in the CNS are similar to the pathology observed in MS [19]. The disease can be adoptively transferred by myelin-reactive $CD4^+$ T cells expanded in vitro.

Ghrelin Suppresses EAE

We have recently tested the effect of ghrelin on EAE [24]. We employed a model in which EAE is actively induced in B6 mice with MOG-derived peptide, MOG_{35-55}. Continuous injections of ghrelin efficiently suppressed the clinical signs of EAE. The effect of ghrelin on EAE was specific, as des-acyl ghrelin, an acyl-modified ghrelin, which lacks an n-octanoic acid on the third serine and consequently cannot bind GHS-R, had no modulatory effect on EAE. These results indicate that ghrelin treatment ameliorates the clinical course of EAE via activation of the GHS-R. Exogenous ghrelin administration suppressed both the induction and effector phases of EAE. Unexpectedly, histological assessment revealed that the infiltration of inflammatory cells in the CNS was not dramatically reduced when compared to the difference in clinical disease severities.

We therefore next tried to understand the mechanisms of ghrelin-mediated suppression of EAE. Since ghrelin is the most potent NPY releasing hormone and NPY suppresses EAE by a Th2 bias, we examined whether ghrelin affects EAE through NPY. Neither the proliferative response nor the IFN-γ (gamma), IL-17 and IL-4 cytokine production by MOG_{35-55}-primed T cells was significantly altered

comparing ghrelin- and sham-treated mice, indicating that the suppression of EAE mediated by ghrelin is not dependent on an NPY effect. Furthermore, transfer of T cells from ghrelin-treated mice resulted in a disease similar to that resulting from transfer of T cells from control mice, suggesting that cells other than T cells are the main targets of ghrelin in the modulation of EAE. However, the reduced expression of proinflammatory cytokines in T cells from the spinal cords of ghrelin-treated EAE mice suggested that ghrelin may affect T cell function either directly or indirectly.

After demonstrating that ghrelin did not suppress the infiltration of inflammatory cells in the spinal cord, we tested how the cytokine milieu was altered in ghrelin-treated mice. Although ghrelin treatment had no effect on the expression levels of IL-4, IL-10, and IFN-γ (gamma) in the spinal cord, spleen, LN, and thymus, levels of TNF-α (alpha), IL-1β (beta), and IL-6 in the spinal cord and TNF-α (alpha) in the spleen were significantly reduced in ghrelin-treated mice compared to saline-treated controls. Because TNF-α (alpha), IL-1β (beta), and IL-6 mRNAs were selectively down-regulated in the spinal cord and we confirmed that ghrelin inhibited proinflammatory cytokine production from the monocytic cell line RAQ 264.7 when stimulated with LPS, we suspected that monocytes could be the potential target cells in ghrelin-mediated EAE suppression. Contrary to our expectation, the expression of proinflammatory cytokines was reduced in spinal cords, particularly in microglia, but not in macrophages. Although monocytes are reported to be the cells primarily affected by ghrelin treatment in animal models of sepsis or arthritis, microglia seem to be an important target in EAE. In fact, LPS-stimulated production of proinflammatory cytokines by microglia isolated from naive mice was reduced when the microglia were cultured in the presence of ghrelin in vitro, indicating that ghrelin directly affects microglial function. Since decreased expression of inflammatory cytokines was also observed in infiltrating T cells in the spinal cords and macrophages in the spleen, ghrelin probably affects not only microglia but also other cells including macrophages and T cells, as described previously.

Microglia in Neuroinflammation

Microglia are glial cells thought to be derived from hematopoietic cells in the CNS [25]. Microglia are a double-edged sword and have both proinflammatory and anti-inflammatory functions (Fig. 14.2) [26]. Under physiological conditions, microglia play an important role as a scavenger cells in the homeostasis of the CNS. However, microglia are an important source of inflammatory cytokines in pathogenic states such as inflammation. Microglia contribute to inflammation through antigen presentation and cytokine/chemokine secretion. Microglia produce Th1-promoting cytokines such as IL-12 as well as inflammatory cytokines such as IL-1β (beta), TNF-α (alpha) and IL-6 [27]. These cells also produce nitric oxide (NO) and glutamate that damage myelin [28]. In contrast, a protective role of microglia in EAE has been also reported [29]. Microglia contribute not only to the clearance of damaged tissue but

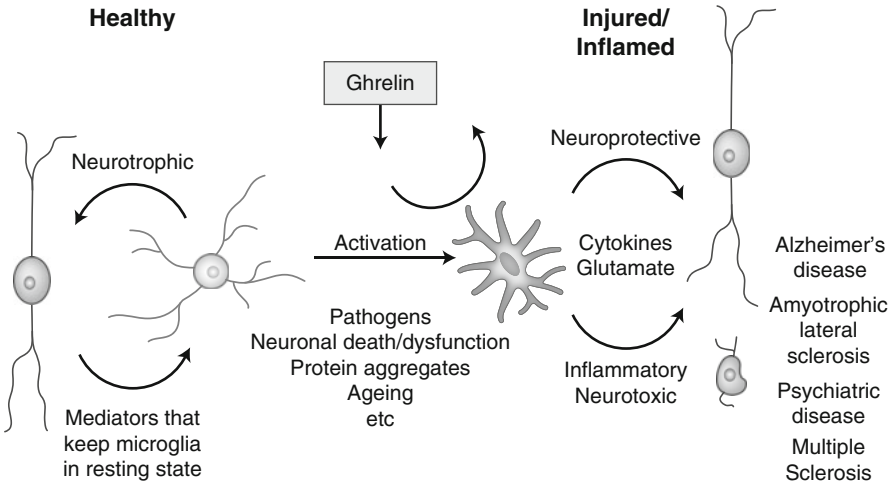

Fig. 14.2 Microglial activation in health and disease. Microglia are a double-edged sword, which have both proinflammatory and anti-inflammatory functions and are thought to be involved in a variety of diseases including MS, neurodegenerative diseases, and psychiatric disorders. Ghrelin suppresses the inflammatory response of microglia and, therefore, ghrelin may be useful for the inhibition of these diseases

also to remyelination, as remyelination is impaired in the absence of microglia and inflammatory cytokines such as IL-1β (beta) and TNF-α (alpha) [30–32]. Microglia are also able to produce neurotrophic factors such as BDNF, PDGF and GDNF [33]. The dual role of microglia may be explained by the existent of different types of microglia, one with inflammatory activity, the other with protective activity just as for classical and altered macrophages. In fact, microglia pretreated with IL-4 induced neurogenesis and oligodendrogenesis in vitro [34, 35]. Interestingly, chronic MS lesions are dominated by activated microglia/macrophages without a prominent lymphocyte infiltration, suggesting an important contribution of innate immunity to the progressive stage of MS [36]. Furthermore, the transition from the RR to the secondary progressive phase of MS has been linked to changes in the peripheral innate immune system [37–40]. These results suggest that targeting innate immune cells may provide a new strategy for the treatment of MS, particularly for the SP phase.

More recently, the involvement of proinflammatory cytokines has been thought to be important not only in inflammation but also in neurodegenerative diseases including Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease and even in psychiatric disorders such as depression. Therefore, to understand the regulatory mechanisms of proinflammatory cytokine production in microglia may be important in the control of a variety of neurological and mental diseases. Ghrelin has been shown to decrease upon aging in association with an increase in inflammatory cytokine production [41]. Micoglial activation with aging also has been reported both in human and experimental animals, although it is difficult to

discriminate whether microglia react to aging-related changes or are directly affected by the aging process [42]. The decrease of ghrelin production upon aging may influence the age-related activation of microglia.

Future Directions

The initial study of the effect of ghrelin on the immune system demonstrated immune-enhancing as well as antitumor effects, whereas recent studies show anti-inflammatory functions in vitro and in vivo. In both cases, however, the effects of ghrelin on the immune system seem to be beneficial to our body, since the former study indicates that ghrelin potentiates immune functions in health and the latter studies show that ghrelin suppresses excessive immune reactions such as sepsis or autoimmune disease including arthritis and encephalomyelitis. Therefore, ghrelin might play a protective role for our body either by enhancing or inhibiting immunity depending on the situations.

Ghrelin is currently undergoing clinical trials for several diseases including cardiac failure, chronic obstructive pulmonary disease and anorexia nervosa [43]. Proinflammatory cytokines are up-regulated in patients with cognitive heart failure, particularly those with cardiac cachexia, and have been implicated in the pathophysiology of this disease. For these patients, ghrelin's anti-inflammatory effect may be beneficial in addition to its orexigenic effect. In addition, our findings that ghrelin is able to suppress microglial activation highlight the potential of ghrelin to treat disorders to which microglial activation contributes such as neurodegenerative diseases and psychiatric disorders.

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Chapter 15
Physiological Roles of Des-Acyl Ghrelin

S.J.C.M.M. Neggers, Riccarda Granata, Ezio Ghigo, and Aart J. van der Lely

Abstract Ghrelin circulates into two different forms: (1) acylated ghrelin (AG) which holds an *n*-octanoic acid at its third serine residue, and (2) des-acyl ghrelin (DAG). AG, but not DAG, binds the GH secretagogue type 1a receptor (GHS-R1a) and stimulates secretion of GH and other pituitary hormones.

Accumulating evidence indicate that both AG and DAG have non-GHS-R1a-mediated biological activities and particularly exert a significant role in the fine-tuning of insulin secretion and glucose metabolism, sometimes acting as agonists others as antagonists. DAG promotes insulin secretion from β-cell lines and enhances the portal insulin response following an intravenous glucose tolerance test in rats. In addition, DAG has been shown to inhibit in vitro hepatic glucose output, suggesting that at least part of the beneficial effect on glucose could also be secondary to an insulin-independent mechanism. Finally, recent results suggest that DAG has also proliferative and protective effects on β-cells. Transgenic mice over-expressing DAG display reduced fat mass and blood triglycerides. Also, the coadministration of AG and DAG reduces plasma FFA in GHD patients. Finally, results of recent preliminary experiments on circulating angiogenic cells (CAC) suggest that DAG may beneficially impact the vascular remodeling process, which is known to be impaired in type 2 diabetes patients.

S.J.C.M.M. Neggers, M.D., Ph.D.
Section of Endocrinology, Department of Medicine, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands

R. Granata, Ph.D. • E. Ghigo, M.D.
Division of Endocrinology, Diabetology and Metabolism, Molinette Hospital, University of Torino, Torino, Italy

A.J. van der Lely, M.D., Ph.D. (✉)
Department of Internal Medicine, Erasmus University MC, Room D-437, P.O. Box 2040, Rotterdam 2662 CW, The Netherlands
e-mail: a.vanderlelij@erasmsumc.nl

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In this chapter, we summarize the data that indicate that DAG exerts several important (metabolic) actions that should separate DAG from AG and make DAG a signaling factor with its own intrinsic role in many metabolic processes.

Introduction

Ghrelin is a 28 amino acid peptide produced by the stomach and circulates into two different forms: (1) acylated ghrelin (AG) which holds an *n*-octanoic acid at its third serine residue, and (2) des-acyl ghrelin (DAG) [1]. In addition, lower amounts of ghrelin have been detected in a wide variety of cells and tissues including the pancreatic β-cells. AG, but not DAG, binds the GH secretagogue type 1a receptor (GHS-R1a) and stimulates secretion of GH and other pituitary hormones [1].

Accumulating evidence indicate that both AG and DAG have non-GHS-R1a-mediated biological activities and particularly exert a significant role in the fine-tuning of insulin secretion and glucose metabolism, sometimes acting as agonists others as antagonists [2–6]. Earlier studies have shown that a single administration of AG to humans significantly induced an increase in plasma glucose that was clearly prevented by coadministration of DAG [2].

Although no change on plasma insulin was noted following acute administration in humans, in vitro and animal data indicate that DAG may also act as an insulin secretagogue: DAG promotes insulin secretion from β-cell lines and enhances the portal insulin response following an intravenous glucose tolerance test in rats [7]. In addition, DAG has been shown to inhibit in vitro hepatic glucose output, suggesting that at least part of the beneficial effect on glucose could also be secondary to an insulin-independent mechanism [8]. Finally, recent results suggest that DAG has also proliferative and protective effects on β-cells [9, 10].

Less is known about effects of DAG on fat and lipid metabolism but available data suggest beneficial effects of DAG administration: transgenic mice overexpressing DAG display reduced fat mass and blood triglycerides [11], the coadministration of AG and DAG reduces plasma FFA in GHD patients [5]. Also, results of recent preliminary experiments on circulating angiogenic cells (CAC) suggest that DAG may beneficially impact the vascular remodeling process, which is known to be impaired in type 2 diabetes patients [12].

Taken all together, data indicate that DAG exerts several important (metabolic) actions that should separate DAG from AG and make DAG a signaling factor with its own intrinsic role in many metabolic processes.

Background and Rationale

AG displays strong growth hormone (GH) releasing activity mediated by the GH secretagogue type 1a (GHS-R1a) receptor [1], initially identified as the receptor of a family of synthetic peptidyl and nonpeptidyl GH secretagogues [13], This receptor

is mainly concentrated in the hypothalamus-pituitary unit and is also distributed in other central and peripheral tissues. In contrast to AG, DAG is not able to bind the GHS-R1a receptor or induce GH secretion [1], and partly because of that, it had been initially considered to be devoid of any physiological activities.

Apart from inducing GH secretion, the ghrelin system has been shown to display a wide variety of endocrine and non-endocrine activities including stimulation of prolactin (PRL) and ACTH secretion, control of appetite and food intake, control of gastric motility and acid secretion, modulation of the pancreatic exocrine and endocrine function, influence of sleep and behavior, modulation of the immune system, and effects on the cardiovascular system (see for reviews [14–17]). Today, accumulating evidence indicate that although DAG has no direct effect on GH secretion and other pituitary function, it is involved in some of the non-endocrine activities of the ghrelin system, particularly those at the metabolic level, either agonizing or antagonizing AG effects.

Ghrelin secretion is pulsatile, plasma levels peak before meals and fall to trough basal levels within an hour after eating [18], suggesting that ghrelin contributes to meal initiation and/or metabolic preparation for nutrient ingestion. Basal total ghrelin levels are usually decreased in obesity and increase back toward normal values after weight loss [19], but little is known about the regulation of AG vs. DAG in obesity and insulin resistance conditions. Obese subjects have been reported to present lower DAG levels than normal weight subjects whereas the AG levels are similar, indicating that obesity might be correlated with a relative DAG deficiency [20]. It has been also observed that insulin-resistant obese subjects have elevated AG/DAG ratio when compared to insulin-sensitive obese subjects [21–23], suggesting that a dysregulation of the AG/DAG ratio might be associated with the development of insulin resistance in obese individuals.

Effects of AG and DAG on Glucose Metabolism

The role of ghrelin in its acylated form in the regulation of glucose metabolism had been predicted by earlier studies showing that chronic treatment with synthetic GH secretagogues worsened insulin sensitivity and induced hyperglycemia in healthy individuals [24, 25], and had later been shown to be independent of the GH-releasing activity, particularly because the metabolic effects were observed in subjects pretreated with a GH receptor antagonist [26]. Several studies undertaken during the past 5 years have documented this role, particularly with regards to participation of AG vs. DAG.

A first clinical study has shown that the i.v. injection of 1 μg/kg AG to healthy subjects induced a prompt and significant increase in glycemia and a decrease in circulating insulin levels [27]. These changes were observed along with increases in GH and persisted over 2 h after the AG administration in contrast with a more transient increase in GH levels, suggesting that effects on glucose and insulin are non-GH-mediated.

The effects on glucose and insulin of a single i.v. administration of 1 μg/kg DAG have been subsequently investigated in healthy subjects [2] and compared to administration of AG alone as well as to coadministration of AG and DAG. In this study, GH, PRL and ACTH were found to be unchanged following administration of DAG which also did not affect the neuroendocrine response to AG. As expected, glucose levels increased and insulin levels decreased following administration of AG. No change was noted following administration of DAG in either parameter, but clearly, the coadministration of DAG was able to antagonize the AG-related rise on glucose. The beneficial effects of the combined administration of DAG and AG on glucose levels have been also confirmed in a subsequent study performed in GHD patients [6].

A recent in vitro work has shown that either AG or DAG promotes proliferation of pancreatic β-cells showing no GHS-R1a receptor and are able to stimulate insulin secretion from these cells [9]. Moreover, the same authors reported recently that UAG has protective effects in STZ-treated rats, both on sparing insulin secretion and mortality [28]. Either peptide also inhibits apoptosis in β-cells and human islets of Langherhans induced either by serum starvation or cytokines whose synergism is known to be a major cause of β-cell destruction in type 1 diabetes, these effects are mediated by the cAMP/protein kinase pathway. Measurements of glucose output by primary hepatocytes in culture have been shown to be dose-dependently stimulated by AG and inhibited by DAG [8], suggesting that the glucose response could reflect a modulation of an insulin-independent hepatic phenomenon. In line with clinical data described above, this study has also shown that DAG counteracted the stimulatory effect of AG and that of glucagon on glucose release.

Overall, these data indicate that both AG and DAG play a role in the regulation of glucose metabolism in humans, and that under pharmacological concentrations, AG may act as a diabetogenic factor, while coadministration of AG and DAG may improve insulin sensitivity. Data also suggest that these effects are independent from the GH-releasing activity of AG and are likely mediated by a receptor distinct from the GHS-R1a, yet unidentified. Based on this background, further pharmacological work has been undertaken including investigation of continuous administration in humans and animal models of diabetes.

Effects of AG and DAG on Fat Metabolism and Energy Balance

Several studies have shown that AG stimulates food intake by central mechanisms, this effect being indeed mediated by the GHS-R1a and depends upon the *n*-octanoyl acylation of ghrelin. In humans, administered AG induces body weight gain and adiposity [29–31]. Preliminary in vitro studies suggest that both AG and DAG have peripheral effects on adipose tissue: both peptides have been shown to promote bone marrow adipogenesis [32] and inhibit isoprotenerol-induced lipolysis in rat adipocytes [33]. Recent studies performed in mice have shown that, in contrast to AG, centrally or intraperitoneally administered DAG induces a negative balance by decreasing food

intake and delaying gastric emptying [34]. Consistent with these results, transgenic mice that over-expressed DAG showed reduced body weight and food intake when compared to non-transgenic littermates [11]. In humans, preliminary data also suggest effects on lipid metabolism; the coadministration of AG and DAG reduces plasma FFA in GHD patients [6].

Effects of AG and DAG on Vascular Remodeling

AG is a diabetogenic and orexigenic gastric polypeptide when administered exogenously [35]. These properties are not shared by the most abundant circulating form, which is DAG. An altered UAG/AG profile together with an impairment of circulating endothelial progenitor cell (EPC) bioavailability was found in diabetes. Based on previous evidence for the beneficial cardiovascular effects of AG and DAG, Togliatto and coworkers investigated the potential of AG and DAG to revert diabetes-associated defects [12]. They studied healthy human subjects, individuals with type 2 diabetes, and ob/ob mice. Both AG and DAG were infused. They evaluated EPC mobilization in patients and mice. The underlying molecular mechanisms were investigated in bone marrow stromal cells. Recovered EPCs were also used to study their potential activity on senescence regulatory pathways and for NADPH oxidase activation by knocking down p47phox and Rac1. DAG modulation of human EPC vasculogenic potential was investigated. Interestingly, neither AG nor UAG had any effect in healthy subjects. However, systemic administration of DAG, but not AG, prevented diabetes-induced EPC damage by modulating the NADPH oxidase regulatory protein Rac1 and improved the vasculogenic potential both in individuals with type 2 diabetes and in ob/ob mice [12]. In addition, unlike AG, DAG facilitated the recovery of bone marrow EPC mobilization. Crucial to EPC mobilization by DAG was the rescue of endothelial NO synthase (eNOS) phosphorylation by Akt, as DAG treatment was ineffective in eNOS knockout mice [12]. These data suggest that DAG plays an AG independent role in vascular remodeling.

How can DAG Exert Any Actions Without Binding to Ghrelin Receptor?

To explain the observations described above, one might wonder how DAG can have any effects at all, as it cannot bind to the GHSR-1a. One possible explanation is that DAG has its own receptor that translates its presence into the observed signals and actions. Several studies are noteworthy in that respect. In several studies, the existence of a non-GHS-R1a that can bind DAG and/or AG is reported. For example, Muccioli and coworkers showed that DAG, AG, short ghrelin fragments, and synthetic GHS act directly as antilipolytic factors on white adipose tissue through binding to a specific receptor which is distinct from GHS-R1a [33]. Also, the study by

Togliatto et al. discussed above, consistently showed that EPCs specifically express DAG-binding sites, not recognized by AG [12]. Perhaps the most compelling demonstration of the existence of a DAG receptor comes from a comparative study in wild-type and GHS-R knockout mice where the effects of peripheral and central administration of AG and DAG on food-intake were measured [36]. Peripheral treatment with AG increased feeding in wild-type, but not in GHS-R knockout mice, whereas peripheral DAG had no effect on food intake in either genotype. By contrast, central administration of DAG increased food-intake in both wild-type and GHS-R knockout mice, but AG was only effective in wild-type mice.

Halem et al. reported the effects of BIM-28163 administration, which fully antagonizes GHS-R1a by binding to but not activating the receptor [37]. They demonstrated that BIM-28163 blocks ghrelin activation of GHS-R1a, and inhibits ghrelin-induced GH secretion in vivo. Unexpectedly, BIM-28163 acts as an agonist with regard to stimulating weight gain, whereas weight loss was anticipated. These results clearly suggest the presence of an unknown ghrelin receptor that modulates ghrelin actions on weight gain. In keeping with their results on growth hormone (GH) secretion, BIM-28163 acts as an antagonist of ghrelin-induced Fos protein immunoreactivity (Fos-IR) in the medial arcuate nucleus, an area involved in the ghrelin modulation of GH secretion. However, in the dorsal medial hypothalamus (DMH), a region associated with regulation of food intake, both ghrelin and BIM-28163 act as agonists to upregulate Fos-IR. The observation that ghrelin and BIM-28163 have different efficacies in inducing Fos-IR in the DMH, and that concomitant administration of ghrelin and an excess of BIM-28163 results in the same level of Fos-IR as BIM-28163 administered alone, may demonstrate that in the DMH both ghrelin and BIM-28163 act via the same receptor. If so, it is unlikely that this receptor is GHS-R1a. Collectively, our findings suggest that the action of ghrelin to stimulate increased weight gain may be mediated by a novel receptor other than GHS-R1a, and imply that GHS-R1a may not be the appropriate target for anti-obesity strategies [37].

Another attempt to study the AG-independent effects of DAG was reported by Delhanty et al. [38]. There is indirect evidence that DAG alters lipid and glucose homeostasis in vivo, but no direct evidence that it can regulate metabolic pathways that control these processes or of the cellular mechanism(s) involved [9, 28]. To address the hypothesis that DAG can modulate metabolic pathways relevant to insulin sensitivity, Delhanty used transcriptome-wide expression profiling of liver, muscle, and white adipose tissue (WAT). The study examined the acute effects of DAG to dissect its direct effects on specific tissues, including possible signaling pathways. To test the hypothesis that DAG acts independently of the GHS-R1a, they examined the effects of DAG on tissues in GHS-R knockout mice [39]. Delhanty and coworkers also tested the hypothesis that AG interacts with DAG in regulating metabolic pathways by examining the effects of DAG in wild type mice. They observed that DAG regulates genes involved in lipid and carbohydrate metabolic pathways in all three tissues in a direction that indicates an overall improvement in metabolic profile, independently of the GHS-R [38].

How can DAG Exert Any Actions Without Binding to Ghrelin Receptor?

Des-acyl ghrelin seems to have effects in vitro and in vivo, both in laboratory animals and in men. These effects often antagonize the effects of ghrelin, especially on changes in body composition and insulin–glucose interactions. However, des-acyl ghrelin also has effects under experimental conditions where no effects of ghrelin can be observed. Of potential clinical importance are the positive effects of des-acyl ghrelin on glucose handling and cardiovascular repair mechanisms. Finally, in some conditions, des-acyl ghrelin and ghrelin seem to have effects that are identical, instead of opposite. As there is no des-acyl receptor found to date, much in vitro and in vivo research is necessary to unravel this complex but fascinating system of one gene that produces products that in some cases can mimic each other, or in other instances be antagonistic.

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Chapter 16
The Ghrelin Receptor (GHSR1a), But Not Ghrelin, Is Essential for Dopamine Receptor-2 (DRD2) Agonist-Induced Anorexia

Andras Kern and Roy G. Smith

Abstract It appears paradoxical that ghrelin receptor (GHSR1a) is expressed in areas of the brain where ghrelin is not produced and where exposure to endogenous ghrelin is unlikely. We show that GHSR1a serves important functional roles in the absence of ghrelin. Non-ghrelin occupied GHSR1a (apo-GHSR1a) modifies canonical dopamine signaling through dopamine receptor-2 (DRD2) by formation of GHSR1a:DRD2 heteromers in native brain tissue. Apo-GHSR1a, but not ghrelin, is essential for the anorexigenic of a DRD2 agonist. These findings illustrate that in neurons expressing both GHSR1a and DRD2, GHSR1a allosterically modifies dopamine signaling and dopamine function. Most importantly, a GHSR1a selective antagonist blocks dopamine signaling in neurons containing both DRD2 and GHSR1a. The therapeutic implications of these results are profound, because by designing agents to selectively target GHSR1a:D2R or GHSR1a:D1R dimers, pharmacological manipulation of dopamine signaling can be targeted to subsets of neurons that co-express GHSR1a and DRD2 without affecting signaling in neurons expressing DRD2 alone. This approach of selective fine-tuning of dopamine signaling provides exciting opportunities for designing next generation therapeutics for feeding disorders and psychiatric disorders associated with abnormal dopamine signaling.

Co-expression of GHSR1a with DRD2 and DRD1 Modifies Dopamine Signal Transduction

Despite the broad distribution of ghrelin receptor (GHSR1a) in the brain, with exception of low levels in the arcuate nucleus, endogenous ghrelin is undetectable [1–3], implicating modification of receptor signaling by interactions of unliganded

A. Kern, Ph.D. • R.G. Smith, Ph.D. (✉)
Department of Metabolism and Aging, The Scripps Research Institute, Florida, Jupiter, FL, USA
e-mail: akern@scripps.edu; rgsmith@scripps.edu

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GHSR1a (apo-GHSR1a) with other brain specific neurotransmitter receptors. By generating transgenic mice where *ires-tauGFP* was knocked-in to the *ghsr* locus so the mice produce a bi-cistronic mRNA that yields GHSR1a and tau-GFP; hence, GHSR1a can be localized to specific neurons by GFP fluorescence. By combining GFP fluorescence with specific antibodies to DRD1 and DRD2 we identified subsets of GHSR1a expressing neurons that express either DRD1 or DRD2 [4, 5].

To determine if co-expression of each dopamine receptor with GHSR1a modified dopamine signal transduction, GHSR1a was expressed with DRD1 and DRD2 in HEK293 cells. In each case canonical dopamine signaling was modified [4, 5]. For example, when dopamine receptor-2 (DRD2) is expressed alone dopamine treatment suppresses cAMP accumulation without affecting $[Ca^{2+}]_i$; however, when GHSR1a and DRD2 are co-expressed, dopamine treatment causes mobilization of $[Ca^{2+}]_i$. This effect is inhibited by PTX and by Gβγ antagonist consistent with regulation by βγ subunits liberated from $G\alpha_{i/o}$. Because GHSR1a and DRD2 co-localize in the hypothalamus, we tested for noncanonical DRD2 signaling in primary cultures of hypothalamic neurons. When these neurons were treated with the DRD2 agonist quinporole rapid Ca^{2+} transients were induced consistent with noncanonical DRD2 signaling. Quinporole was removed by washing and the same neurons treated with ghrelin. Ghrelin immediately produced a Ca^{2+} response confirming that GHSR1a and DRD2 were co-expressed in these neurons [5].

Extraordinary low levels of GHSR1a are present at in native tissues [6]. However, when GHSR1a is expressed in heterologous systems at levels greater than in native tissues it exhibits basal activity. Accordingly, it is widely believed that GHSR1a basal activity has physiological relevance. Although we did not share this belief, it was important to rule out this possible mechanism that might explain modification of canonical DRD2 signaling by GHSR1a. Perhaps, the closest example of basal activity of one GPCR modifying signaling of another in vitro is the report of co-expression of mGlu1a with $GABA_B$ receptor [7]. Like GHSR1a, mGlu1a couples to $G\alpha_q$, and like DRD2, $GABA_B$ couples to $G\alpha_{i/o}$. Co-expression results in a synergistic increase in GABA-induced mobilization of $[Ca^{2+}]_i$ and the authors concluded that augmentation of $[Ca^{2+}]_i$ mobilization was caused by integration of $[Ca^{2+}]_i$ responses as a consequence of mGlu1a basal activity. However, in cells co-expressing GHSR1a with DRD2 there was no evidence for receptor cross talk or augmentation of $[Ca^{2+}]_i$ in response to agonists of either receptor. The possibility that basal activity of GHSR1a explains modified dopamine signaling was eliminated by a series of additional experiments: blocking GHSR1a signaling with $G\alpha_q$ siRNA failed to attenuate dopamine-induced mobilization of $[Ca^{2+}]_i$; substituting GHSR1a with GHSR1a point mutants illustrated that basal activity of the mutants did not correlate with mobilization of $[Ca^{2+}]$ by dopamine; when the closely related motilin receptor that couples through $G\alpha_q$ [8] was co-expressed with DRD2, dopamine treatment did not induce a Ca^{2+} response [5]. These data suggested that GHSR1a expression with DRD2 resulted in noncanonical dopamine/DRD2 signal transduction because of allosteric interactions between GHSR1a and DRD2 protomers. Further evidence for molecular interactions was illustrated by showing that a GHSR1a inverse agonist or

neutral antagonist did not disrupt heteromer formation but abolished dopamine signaling; similarly, a DRD2 inverse agonist attenuated ghrelin-induced Ca^{2+} signaling.

GHSR1a and DRD2 Form Heteromers

To test whether modification of signal transduction is a consequence of physical interactions between GHSR1a and DRD2, we first performed time resolved fluorescence resonance energy transfer (Tr-FRET) studies in HEK293 cells [5]. Tr-FRET is ideal for monitoring cell surface protein–protein interactions of receptors at physiological concentrations [9, 10]. SNAP-tag was introduced at the GHSR1a N-terminus. GHSR1a forms homodimers on the cell plasma membrane. When SNAP-GHSR1a was expressed with DRD2 at an equimolar ratio the Tr-FRET signal generated by GHSR1a:GHSR1a homomers is reduced to 62 ± 10%, supporting formation of GHSR1a:DRD2 heteromers. A control GPCR, RXFP1, co-expressed with SNAP-GHSR1a failed to attenuate the Tr-FRET signal. As a confirmational test of GHSR1a and DRD2 heteromer formation, CLIP-tagged GHSR1a and SNAP-tagged DRD2 were prepared and the results of co-expression examined using confocal microscopy. The CLIP- and SNAP-tagged receptors co-localized on the cell surface. Dose titration experiments illustrated saturable Tr-FRET signals indicating Tr-FRET was explained by specific heteromerization rather than random collisions. When the Tr-FRET data were compared to the magnitude of dopamine-induced $[Ca^{2+}]_i$ mobilization at different ratios of GHSR1a to DRD2, the magnitude of dopamine-induced Ca^{2+} release correlated with the level of GHSR1a:DRD2 heteromers. These results confirmed that alteration of canonical DRD2 G-protein coupling did not require ghrelin and was dependent upon expression of both receptors and formation of GHSR1a:DRD2 heteromers [5].

GHSR1a:DRD2 Heteromers Exist in the Hypothalamus

Reports of GPCR protomers forming heteromeric complexes in vitro are not new [11–13]; nevertheless, because of the dearth of data showing existence of heteromers in native tissue and the lack of functional correlates in animals it is frequently argued that these heteromers are in vitro artifacts and physiologically irrelevant. An exception of course is the $GABA_B$ receptor, where two dissimilar subunits are required for agonist-induced signal transduction in vivo [14].

To address the question of formation of GHSR1a:DRD2 in the brain we applied Tr-FRET assays on plasma membranes isolated from striatum and hypothalamus. Membranes were incubated with red fluorescent ghrelin as the acceptor fluorophore, and a specific monoclonal antibody for DRD2 with terbium cryptate labeled secondary antibody as the donor fluorophore. The Tr-FRET signal obtained from the striatal

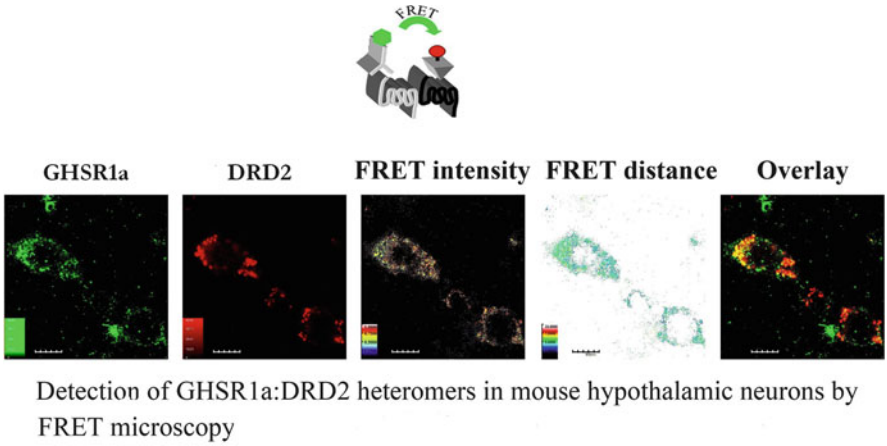

Fig. 16.1 Detection of GHSR1a:DRD2 heteromers in hypothalamus of mouse brain by confocal microscope FRET. GHSR1a was identified using red fluorescent ghrelin; DRD2 with immunofluorescent DRD2 monoclonal antibody (Kern A et al. (2012) Neuron 73(2):317–332). Published by permission of Cell Press

membranes did not reach significance, but hypothalamic membrane preparations exhibited a significant signal. As a test of assay specificity we compared Tr-FRET assays on membranes of brain tissues from wild type (*ghsr*+/+) and *ghsr* knockout mice (*ghsr*−/−). Significantly higher FRET signals were observed in hypothalamus from wild-type mice compared to knockout mice [5].

To more directly test for the existence of GHSR1a:DRD2 heteromers in the brain we assayed brain slices from *ghsr*+/+ and *ghsr*−/− mice using fluorescently labeled red-ghrelin to localize GHSR1a in neurons and a DRD2 monoclonal antibody for localization of DRD2 [5]. As observed with striatal membranes, the FRET signals in the striatum were very weak; hence, heteromerization in striatal neurons could not be documented. In the hypothalamus, confocal FRET analysis showed that GHSR1a and D2R receptors are in close proximity with a relative distance of 5–6 nm (50–60 Å) confirming physical interaction and heteromer formation between natively expressed GHSR1a and DRD2 in the hypothalamus [5] (Fig. 16.1).

Physiological Relevance of GHSR1a:D2R Heteromers in Feeding Behavior

The presence of GHSR1a:DRD2 heteromers in native brain tissue begs the question of whether preventing heteromer formation results in an altered behavioral phenotype. DRD2 affects feeding behavior and mutations in *DRD2* are associated with human obesity [15–18]. Based on such reports illustrating the importance of DRD2

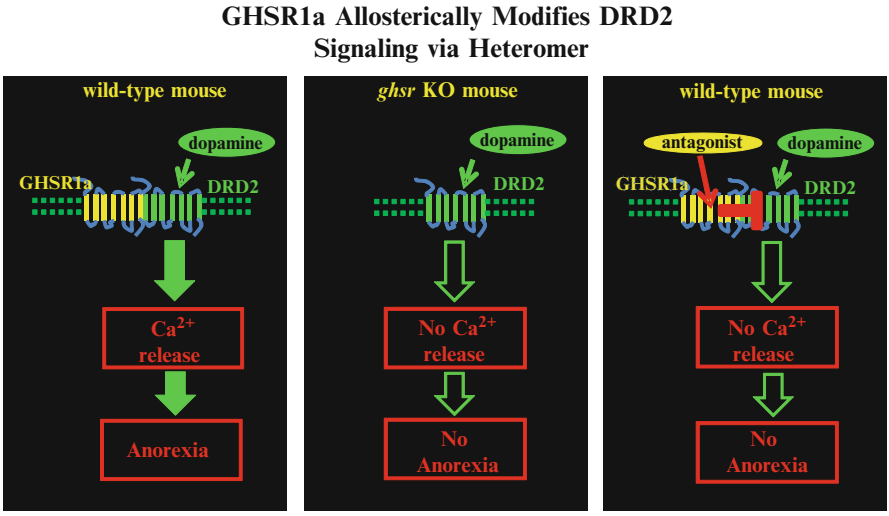

Fig. 16.2 Illustration of the dependence of the anorexigenic effect of a DRD2 agonist on non-agonist occupied GHSR1a (apo-GHSR1a), but not on ghrelin. The DRD2 agonist cabergoline inhibits food intake in wild type mice (*ghsr*+/+) and in ghrelin knockout mice (*ghrelin*–/–). Ghrelin receptor knockout mice (*ghsr*–/–) or wild type mice (*ghsr*+/+) treated with the ghrelin receptor neutral antagonist, JMV2959 are refractory to the anorexic effects of cabergoline (Kern A et al (2012) Neuron 73(2):317–332)

signaling on food intake, we tested the functional significance of interactions between GHSR1a and D2R in vivo by treating fasted *ghsr*+/+ and *ghsr*–/– mice with a DRD2 selective agonist. We selected cabergoline because of its higher affinity for DRD2 than other dopamine or serotonin receptor subtypes. Food-intake was measured in fasted *ghsr*+/+ and *ghsr*–/– mice for up to 24 h following treatment with the DRD2 agonist cabergoline. In *ghsr*+/+ mice food intake was markedly reduced compared to vehicle treated mice, but food intake in *ghsr*–/– mice was unaffected by cabergoline treatment. Hence, DRD2-induced anorexia is dependent upon GHSR1a expression. Intriguingly, in direct contrast to *ghsr*–/– mice, treatment of *ghrelin*–/– mice with cabergoline induced anorexia identical to that observed in wild type mice showing that the anorexigenic effect of the DRD2 agonist is dependent upon GHSR1a, but not ghrelin [5].

In HEK293 cells co-expressing GHSR1a and DRD2 the GHSR1a antagonist JMV2959 blocked DRD2-agonist-induced mobilization of Ca^{2+}. To test whether JMV2959 would block anorexia induced by cabergoline in mice, wild type mice were treated with cabergoline + vehicle, or cabergoline + JMV2959. Intriguingly, JMV2959 blocked cabergoline inhibition of food-intake in *ghsr*+/+ mice [5]. A summary of the collective findings is represented in Fig. 16.2. Putting these findings into the context of attenuated DRD2 signaling being associated with obesity in humans [18], we would predict that GHSR1a antagonists would exacerbate rather than prevent obesity.

Conclusion

By applying confocal microscopy and FRET analysis on brain slices we have shown that GHSR1a:DRD2 heteromers are naturally formed in hypothalamic neurons. Collectively, the results support our hypothesis that in neurons co-expressing GHSR1a and DRD2 allosteric modulation by GHSR1a in the complete absence of ghrelin results in differential response to endogenous dopamine compared to neurons expressing DRD2 alone. Indeed, our results are consistent with the notion that in a subset of neurons the allosteric effect of the GHSR1a is coupled to the simultaneous presence of dopamine to allow selective fine-tuning of neuronal responses to the endogenous neurotransmitter. Intriguingly, through the GHSR1a:DRD2 heteromer, an agonist, inverse agonist or antagonist of one protomer influences the signaling properties of the other. Furthermore, disruption of the active conformation of one protomer by mutagenesis influences the structure of the heteromer and modifies function. Accordingly, the heteromer is a structural base for developing new agents with distinct pharmacological properties capable of discriminating between neurons expressing DRD1, DRD2, DRD1+GHSR1a, and DRD2+GHSR1a. Therefore, our findings reveal new opportunities for selectively modifying dopamine signaling in discrete sets of neurons by targeting heteromers rather than homomers or protomers.

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Index

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