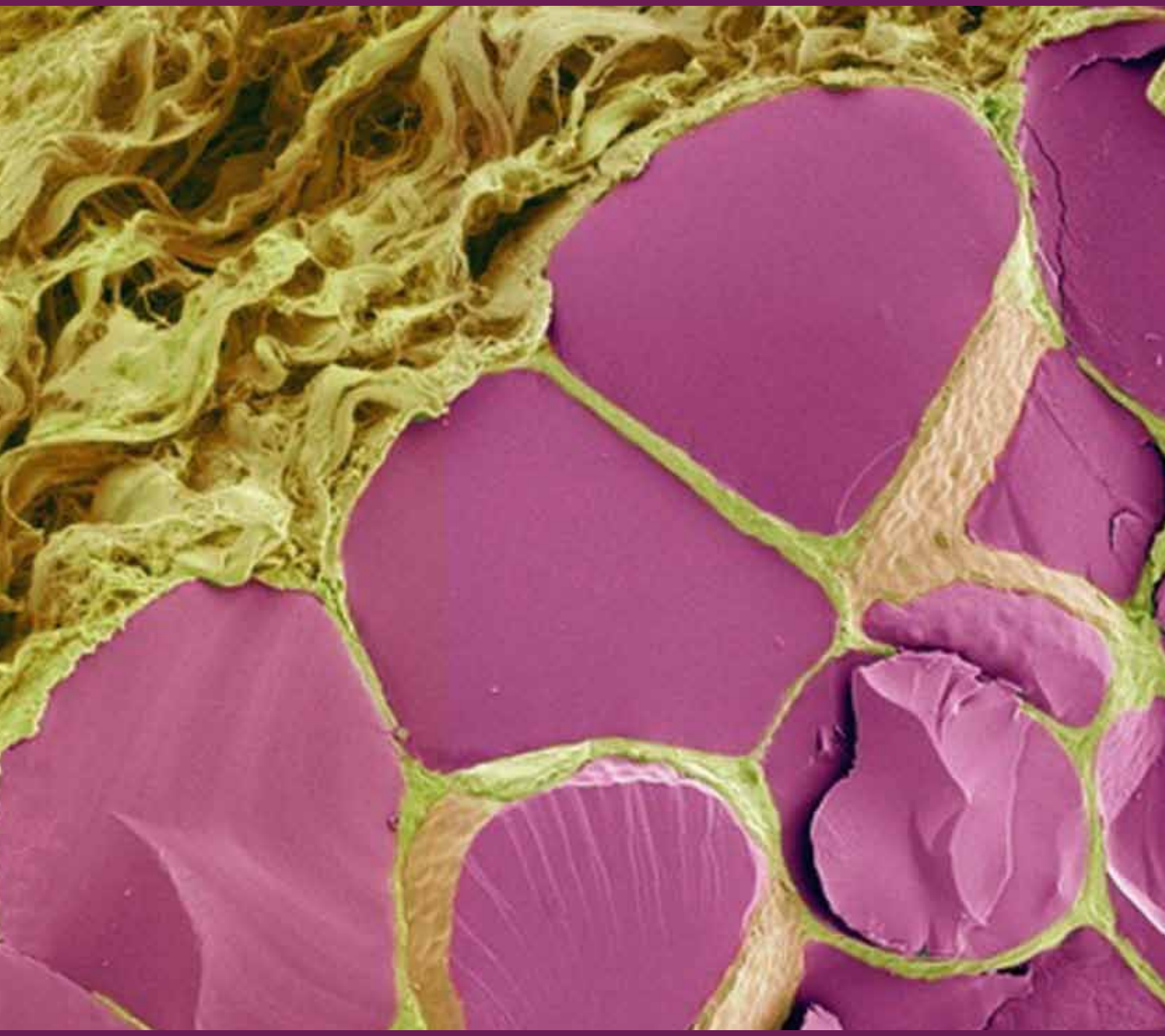
International Journal of Endocrinology

Neuropeptides and Control of Food Intake

Guest Editors: Paolo de Girolamo and Carlos Dieguez



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Editorial Board

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2014, Article ID 910912, 2 pages
http://dx.doi.org/10.1155/2014/910912

Editorial

Neuropeptides and Control of Food Intake

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Received 10 March 2014; Accepted 10 March 2014; Published 2 April 2014

In all vertebrates, food intake is a sophisticated complex of neurohumoral networks that convey signals between the brain and periphery, to modulate energy status. Gut hormones, such as peptide YY, pancreatic polypeptide, glucagon-like peptide-1, oxyntomodulin, and ghrelin, are modulated by acute food ingestion. In contrast, adiposity signals such as leptin and insulin are implicated in both short- and long-term energy homeostases. The mechanisms of action of these substances are similar among vertebrates.

Their regulation might vary with the feeding and reproductive state, and between different tissues and organs, and it might also be affected by environmental parameters. The control of food intake is carried out by short-term and long-term regulation mechanisms. The short-term signals act primarily as determinants of satiety to limit the size of individual meals. Long-term signals communicate total energy stores, integrate over time, and interact with other systems that rely upon the energy status of the organism (e.g., growth, immune function, and reproduction). Both long- and short-term signals interact to influence the behavior and energy balance of the organism. We know that disrupted signaling in many of these systems leads to dramatic changes in feeding behavior and weight gain (or loss). However, fully understanding control of food intake will require knowledge of: (a) which peptides are involved; (b) areas of the central nervous system where this peptides are expressed and (c) assessment of the biological effects of the different neuropeptides in the integrated control of energy and metabolic homeostasis.

In this special issue we selected several papers that carry out a systematic and critical review of some of the topics that following recent developments are currently at the forefront of obesity research. This special issue is particularly timely since it becomes available 20 years after the seminal discovery of leptin in Friedman's lab.

H. M. Zerón et al. provide a deep and comprehensive review of the different gut hormones involved in energy homeostasis; they give an update on the available evidence regarding the interaction between macronutrients and gastrointestinal hormone secretion. Furthermore, they review the available data regarding the evidences postulating a yet uncharacterized production of a putative hormone produced in the foregut of diabetic patients that should act decreasing insulin-sensitivity at peripheral tissues. This topic is of particular clinical relevance since it provides a rationale for the so-called metabolic surgery that has been advocated by some as possible therapy in diabetic patients.

Most of the data gleaned over the last 20 years in energy homeostasis has been focused in the integrated control exerted at hypothalamic level between central and peripheral signals. The latter arises from the gastrointestinal tract, including the pancreas, and the adipose tissue. More recently it has become evident that proteins secreted for other peripheral tissues may also play an important role. Thus a new set of factors so-called myokines, produced by muscle and released into the blood circulation, has emerged as major regulators of energy and metabolic homeostasis. A recently discovered one, irsin, is postulated as a potential drug target due to its marked effects on energy expenditure and therefore body weight. The available data in this topic is reviewed by M. G. Novelle et al., who provide a critical assessment at the available evidences so far and some of the yet remaining

questions regarding the mechanisms involved in its secretion and biological effects.

The intimate relationship between energy status and reproduction has been recognized for many years. Data gleaned more recently have clearly shown that signals, such as leptin and ghrelin, known to be involved in the regulation of energy homeostasis do also play an important role in the control of the hypothalamus-pituitary-gonadal axis. However, the mechanisms involved at central level mediating their effects have only recently become clearer. In this special issue, J. Roa provides an overview of the most recent developments in the field. In particular, a deep mechanistic insight assessing the implication of GnRH, Kiss1, NPY, and POMC neurons as the key factors contributing to the adaptation of the gonadal axis to different metabolic status is put forward.

Anorexia and bulimia nervosa are two clinical entities characterized by abnormal eating behavior. Despite many years of research, the mechanisms involved are far from being clarified. K. Smitka et al. extensively review the involvement of the different neuronal networks implicated in food intake and the available evidences regarding the alteration of different hormones and neuropeptides as potential physiopathological mechanisms in these two diseases. Furthermore they carry out a thorough assessment of the potential involvement of neutralizing autoantibodies against these peptides.

A topic that was neglected for many years but that is now at the forefront of research is the assessment of nutrition and anxiety. M. Murphy and J. G. Mercer review the most recent developments in the field. In particular, they critically review available data on the influence of different dietary components on anxiety-like behavior. Furthermore, they highlight the importance of fetal and neonatal programming and the limitations when comparing the outcomes of trials that involve differences in diet, species, strain, sex, and life stage, coupled with variation in duration, environment, and outcome measure.

Most of the studies regarding body weight homeostasis were focused in the issue of energy intake. Over the last few years considerable attention has been given to the study of mechanisms that promote or are stimulated by palatable food. J. R. Barson et al. addressed this issue by focusing on the two neuropeptides historically related to the lateral hypothalamus. They provide us with a deep insight into the mechanisms by which orexin and MCH promote the intake of palatable and/or caloric food and how the intake of these foods can influence the activity of the neurons producing these neuropeptides. In addition they describe their effects on the different brain area implicated in food reward. ¡ack¿¡title/¿

Acknowledgment

The guest editors wish to thank all authors for their valuable contributions. Without their efforts, this special issue would not have been possible.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 598203, 12 pages
http://dx.doi.org/10.1155/2013/598203

Review Article

Peripheral Pathways in the Food-Intake Control towards the Adipose-Intestinal Missing Link

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Received 26 March 2013; Accepted 16 October 2013

Academic Editor: Paolo de Girolamo

In the physiological state a multitude of gut hormones are released into the circulation at the same time depending on the quality and quantity of the diet. These hormones interact with receptors at various points in the "gut-brain axis" to affect short-term and intermediate-term feelings of hunger and satiety. The combined effects of macronutrients on the predominant gut hormone secretion are still poorly understood. Besides, adipokines form an important part of an "adipoinsular axis" dysregulation which may contribute to β-cell failure and hence to type 2 diabetes mellitus (T2DM). Even more, gestational diabetes mellitus (GDM) and T2DM seem to share a genetic basis. In susceptible individuals, chronic exaggerated stimulation of the proximal gut with fat and carbohydrates may induce overproduction of an unknown factor that causes impairment of incretin production and/or action, leading to insufficient or untimely production of insulin, so that glucose intolerance develops. The bypass of the duodenum and jejunum might avoid a putative hormone overproduction in the proximal foregut in diabetic patients that might counteract the action of insulin, while the early presentation of undigested or incompletely digested food to the ileum may anticipate the production of hormones such as GLP1, further improving insulin action.

1. Introduction

Under steady-state conditions, all ingested fuels (energy intake) are normally metabolized to maintain basic metabolic rate, thermogenesis, and muscle action (energy expenditure). Food intake and energy expenditure can be influenced by environment and lifestyle. This knowledge highlights the importance of understanding the physiological and molecular mechanisms responsible for the final predominated signal of appetite control [1].

All of the peripheral and central processes that make up this highly complex system are subject to individual predisposition through genes. Key peripheral components are the gustatory system, the gastrointestinal tract, pancreas, liver, muscle, and adipose tissue (Figure 1). The aim of this review is to give an insight into the major peripheral signals in the food intake control, viewed in a dynamic context taking into account the major food related signals and to discern possible explanations of the diabetogenic state recovery after weight lose [2].

2. Intestinal Signals

Over 30 different regulatory peptide hormones are secreted in the gut, the largest endocrine organ in the body. Gut nutrient content stimulates several of these hormones which interact with receptors at various points in the "gut-brain axis" to affect short-term and intermediate-term feelings of hunger and satiety [3]. The major gut hormones implicated in appetite control (Table 1) are age, sex, and body mass index (BMI) dependent (Table 2).

By chemo/mechanosensory mechanisms, the gastrointestinal tract sends information to the brain regarding

Table 1: Major peripheral signals involved in food intake regulation.

	Hormone	Site of secretion	Major receptors	Major actions
Intestinal	Amylin	Pancreatic β cells	AMY_{1-3}	Inhibits gastric secretion Delays gastric emptying Decreases blood glucose
	Cholecystokinin	Intestinal Icells	CCK2	Gall bladder contraction Delays gastric emptying Pancreatic enzyme contraction
	Endocannabinoid system	Postsynaptic cell	CB1, CB2	Modulates appetite besides a variety of physiological processes
	Ghrelin	Gastric fundal A cells	GHS–R	Increases gastric motility Growth of hormone release
	Glucagon	Pancreatic α cells	Glucagon	Gluconeogenesis Glycogenolysis
	Glucagon-like peptide-1 (GLP-1)	Gastrointestinal L cells	GLP–1	Glucose-dependent insulin release Delays gastric emptying Vagal and CNS effects
	Glucose-dependent insulinotropic polypeptide (GIP)	K cells in duodenum and jejunum	GIP–R	Stimulates insulin synthesis and secretion
	Oxyntomodulin	Gastrointestinal L cells	GLP–1	Glucose-dependent insulin release Delays gastric emptying Vagal and CNS effects
	Pancreatic polypeptide	Pancreatic PP cells	Y_4	Delays gastric emptying
	Peptide YY (PYY)	Gastrointestinal L cells	Y_2	Delays gastric emptying Vagal and CNS effects
Adipose	Adiponectin	Adipocyte, skeletal muscle, endothelial cells, and cardiomyocytes	AdipoR1 AdipoR2 T-cadherin	Adiponectin, via AMPK phosphorylation, increases insulin sensitivity, fatty acid oxidation and reduces the synthesis of glucose in the liver and other tissues
	Leptin	Adipocyte	LEPR	Increases POMC anorexigenic signals Inhibits NPY, stimulating appetite
	Plasminogen activator inhibitor-1 (PAI-1)	Endothelium, adipocyte	Binds to (tPA)	Inhibitor of fibrinolysis
	Tumour necrosis factor alpha (TNF-α)	Adipocyte	Tumor necrosis factor receptor (TNFR)	Insulin resistance

AMPK: AMP-activated protein kinase, CNS: central nervous system, NPY: neuropeptide Y, POMC: proopiomelanocortin, and tPA: tissue plasminogen activator.

available energy for metabolism. Postprandially, activation of gut mechanoreceptors, changes in circulating nutrient concentration, and release of anorectic gut hormones all lead to a reduction in subsequent feeding [4]. However, apart from traditional homeostatic feedback regulation of energy balance, food appearance, flavor, and availability in addition to social, cultural, and economic influences determine food intake. Importantly, there is also modulation of food intake by hedonic and mnemonic neuronal circuits [5]. The modern consensus is, therefore, that there is interaction between homeostatic and nonhomeostatic inputs, which together lead to coordination in terms of inducing either an orexigenic or anorectic response.

2.1. Amylin. Amylin or islet amyloid polypeptide (IAPP) is a 37-amino acid pancreatic peptide that is cosecreted with insulin. This hormone is a member of the calcitonin family of peptides and is involved in meal satiety signaling [6–8]. As such, amylin and related compounds also inhibit gastric emptying and reduce meal size [9, 10].

Synthetic or naturally occurring amylin agonists have been shown to be more potent and have a longer duration of feeding suppression than amylin itself [11]; one such potent anorectic analog in humans, primates, and rodents is calcitonin of salmon origin (sCT) [12]. This compound irreversibly binds to amylin receptors to produce sustained anorectic responses [13]. Additionally, the anorectic potency of amylin agonists is not dependent on intact vagal afferent signaling [14].

2.2. Cholecystokinin. Cholecystokinin (CCK) is considered a highly selective satiation signal acting over two receptors, the CCK-B, predominantly found in the brain, where the unsulfated tetrapetide CCK-4 is active and the A-type

Table 2: BMI, age, sex, and gut hormones.

	Children				Female							Male		Adults		
Age	9.62 ± 0.42 [15]	9.68±0.29 [15]	24 ± 8 [16]	24 ± 5 [16]	15 ± 1 [17]	41.2 ± 12.9 [18]	53.5±10.8 [19]	14 ± 1 [17]	47.9 ± 7.8 [20]	47.9±7.8 [20]	46.6 ± 13.1 [21]	53.5 ± 10.8 [19]	51 ± 7 [22]	53.5±10.8 [19]	47.1±2.5 [23]	45.0 ± 2 [23]
BMI (kg/m^2)	17.3 ± 0.4 [15]	27.9 ± 1.1 [15]	15.4 ± 1.4 [16]	20.9 ± 1.9 [16] 21.1 ± 0.6 [24]	22.2±0.7 [17]	25.4 ± 4.9 [18]	30.3 ± 6.1 [19]	33.0 ± 3.3 [17]	39.0 ± 3.8 [20]	42.8±3.8 [20]	47.1 ± 88.1 [21]	30.3 ± 6.1 [19]	42.1 ± 7.0 [22]	30.3 ± 6.1 [19]	41 ± 1 [23]	48 ± 1 [23]
CCK (pmol/L)					1.4 ± 0.1 [17]			0.5 ± 0.2 [17]								
Leptin ($\mu g/L$)	3.52±0.85 [15]	31.8 ± 4.0 [25]	2.3 ± 1.3 [16]	12.0±6.73 [16]		18.5 ± 11.6 [18]	26.8 [19]		22.0±12.3 [20]	30.6 ± 11.7 [20]					36.8±2.7 [23]	38.2±2.0 [23]
Ghrelin (pg/mL)	835 ± 47 [15]	664 ± 37 [15]					881.7 ± 413.5 [19]		519 ± 105 [20]	425 ± 91 [20]		764.5 ± 292.5 [19]	632 ± 99 (pmol/L) [22]	817.0 ± 355.5 [19]	263 ± 34 [23]	330 ± 25 [23]
GIP (pmol/L)					37 ± 7 [17]			41 ± 5 [17]								
GLP-1 (pmol/L)									6.12±4.19 [20]	6.02 ± 2.77 [20]	0.65 ± 0.18 (ng/mL) [21]	5 − 10 [26]			8.2 ± 1.5 [23]	5.8 ± 1.4 [23]
PYY (pg/mL)	87.0 ± 7.9 [15]	111.1 ± 11.0 [15]		86 (pg/L) [24]					69.5±41.3 [20]	69.4 ± 44.3 [20]					130 ± 17 [23]	90 ± 19 [23]

BMI: body mass index, CCK: cholecystokinin, GIP: gastric inhibitory peptide, GLP-1: glucagon-like peptide-1, and PYY: peptide YY.

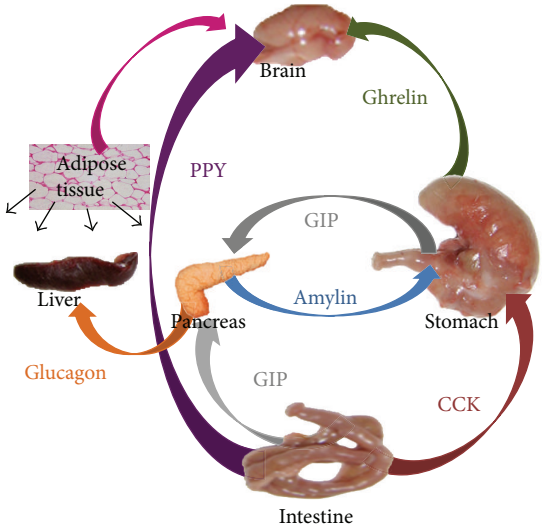

Figure 1: Interacting hormones involved in food-intake control. GIP: glucose-dependent insulinotropic polypeptide; PYY: peptide YY.

receptor of the gastrointestinal tract, where the sulfated CCK-forms (CCK-8-S, CCK-33-S, CCK-39-S, and CCK-58-S) bind. Satiety and meal size limitation are mediated mainly by the CCK-A-receptor [27].

Luminal fat and protein are strong releasers of CCK from enteroendocrine cells. The food intake suppressive effect results from CCK acting in a paracrine fashion on CCK-A receptors located on vagal sensory nerve terminals in the mucosal lamina propia [28–30]. Intact vagal afferent neurons are required for the satiety effects of CCK.

2.3. Endocannabinoid System. There are reports suggesting that the peripheral endocannabinoid system is implicated in the regulation of energy balance. For instance, during periods of fasting, levels of the endocannabinoid and anandamide are elevated in the rat duodenum [31]. Furthermore, in obese rodents, an increase in mRNA for CB1 receptors is observed in the stomach [32] and in the nodose ganglia [33], and endocannabinoid levels in the duodenum, pancreas, and liver are similarly elevated in this animal model [34]. On the contrary, cannabinoid CB1 receptor antagonists reduce food intake and body weight, but clinical use in humans has been limited by effects on the central nervous system (CNS), although there are new options with limited CNS penetration [35].

2.4. Ghrelin. Ghrelin is an orexigenic hormone [36], secreted in the oxyntic gland cells in the mucosa of the stomach, originally isolated from the rat stomach as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R), and has been shown to have a GH-releasing effect [37]. Yet, the ghrelin receptor is expressed by a subset of stomach innervating vagal afferent neurons in the nodose ganglia [38].

Plasma ghrelin concentrations are elevated during a fast. Moreover, plasma ghrelin concentrations display a circadian rhythm: a rise before each meal and a rapid fall after eating. Fasting morning ghrelin concentrations have a negative correlation with fat mass index [39]. On the other hand, diet-induced weight loss in obese individuals increased plasma ghrelin levels [40]. These findings suggest that plasma ghrelin levels may represent a compensatory response to altered energy metabolism. Of note, central and peripheral administration of ghrelin stimulates food intake and body weight gain [37].

2.5. Glucose-Dependent Insulinotropic Polypeptide (GIP). Glucose-dependent insulinotropic polypeptide (GIP) is manufactured and released in the duodenum and proximal jejunum by the K cells. Its plasma concentration increases quickly following food ingestion, stimulating an increase in insulin synthesis and secretion [41]. Carbohydrate, fat, and protein have all been shown to stimulate GIP secretion [42]. Pancreatic and duodenal homeobox-1 (Pdx-1) binds to GIP promoter. This, together with the fact of a remarkable reduction in the number of GIP-expressing cells in Pdx−/− mice [43], suggests that this incretin may play a role in β-cell differentiation.

2.6. Glucagon-Like Peptide-1 (GLP-1). Proglucagon is a 160-amino acid prohormone that is produced in the α cells of the pancreatic islets, the L cells of the distal gut, and within the CNS. Selective posttranslational proteolysis of proglucagon by prohormone convertases 1 and 2 results in the tissue-specific production of a number of biologically active fragments.

GLP-1 is a potent insulin secretagogue that is secreted, in response to ingested nutrients. GLP-1 and related agonists, such as exendin-4, have been demonstrated to reduce food intake by slowing gastric emptying, reducing meal size, and promoting feelings of satiety [44, 45]. The reductions in food intake by these compounds appear to be peripherally mediated, as they are dependent on intact vagal afferent signaling [46]. The importance of the vagus nerve in mediating the proximal-distal loop was elucidated from the evidence that GLP-1 secretion is enhanced when the fat is administered into the duodenum or when the GLP-1 secretion, in response to the infusion of physiological concentration of GIP, was completely abrogated by vagotomy [47].

2.7. Oxyntomodulin. Another product of the tissue-specific differential cleavage of proglucagon is oxyntomodulin (OXM), a hormone cosecreted with GLP-1 and $PYY_{3\text{-}36}$ into the circulation by intestinal L-cells after nutrient ingestion [48]. OXM is a satiety signal through GLP-1R [36, 49] and administration reduces energy intake in both rodents and humans [50, 51]. OXM levels are increased after gastric bypass surgery.

2.8. PYY. PYY is a 36-amino acid peptide, which belongs to the pancreatic polypeptide (PP) family, which also includes NPY. All these bind to G-protein coupled receptors Y_1, Y_2, Y_4, Y_5, and Y_6, displaying promiscuity in their interactions with these receptors by virtue of their shared hair-pin-fold motif structure [3].

PYY is produced by the L cells of the gut, with highest concentrations found in the large bowel and the rectum [52]. Two endogenous forms, PYY_{1-36} and PYY_{3-36}, are released postprandially into the circulation. PYY_{3-36}, which acts mainly via the Y_2 receptor, is further produced by cleavage of the Tyr-Pro amino terminal residues of PYY_{1-36} by the enzyme dipeptidyl peptidase IV (DPP-IV). PYY_{1-36} predominates in the circulation in the fasted state, whereas PYY_{3-36} is the major circulating form postprandially. Following a meal, circulating levels of PYY_{3-36} rise within 15 min, peak at approximately 90 min and remain elevated for up to 6 hours [53]. The magnitude of the rise in PYY_{3-36} is in proportion to the calories ingested [54]. When exogenously administered intravenously, its circulating half-life is approximately 8 min [43].

Initial postprandial release of PYY_{3-36} is likely to be under neural control, and further release of PYY_{3-36} is observed when the nutrients arrive in the distal gut, particularly stimulated by a high fat diet [55]. The protein content of the diet is thought to be influential for delayed PYY_{3-36} release approximately 2 hours postprandially [56]. Besides a direct central action, PYY_{3-36} is likely to affect appetite via its effects on gut motility, leading to a sensation of fullness and satiety [57].

3. Adipose Signals

Adipokines form an important part of an "adipoinsular axis," dysregulation of which may contribute to β-cell failure and hence to T2DM. While some adipokines have beneficial effects, others have detrimental properties depending on the predominant intracellular signalling pathways [58]. The major cause of T2DM could be a human metabolic zwitterion-like molecule, with positive or negative effects over the beta cell depending on its state of activation.

3.1. Adiponectin. Unlike many other adipokines, adiponectin has beneficial effects improving insulin sensitivity and vascular function, thus being both antidiabetic [59] and antiatherogenic [60]. Opposite to other adipokines the circulating levels are decreased when the BMI is higher. The loss in body weight by adiponectin is mainly due to stimulation of energy expenditure [61].

Two adiponectin receptors AdipoR1 and AdipoR2 that exhibit 67% homology have been cloned. Many of the effects of the adiponectin-AdipoR interaction have been suggested to be mediated by $5'$ AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor a (PPARa), and p38 mitogen-activated protein kinases (MAPK) [62].

3.2. Leptin. Leptin is thought to signal longer-term energy status. This hormone engages a number of intracellular pathways, including those associated with cyclic adenosine monophosphate (cAMP), MAPK, phosphatidylinositol $3'$-kinase (PI3 K), and signal transducer and activator of transcription 3 (STAT3) [63, 64].

In contrast to adiponectin, serum concentration of circulating leptin is elevated in obesity. Thus, probably there is a decrease in response to leptin [61]. Although leptin showed a great potential in preclinical studies, it was usefulness in clinical trials [65]. In eating disorders the results are controversial [66].

In the hypothalamic arcuate nucleus there are two types of neuronal populations with high levels of expression of the leptin receptor (LEPR), proopiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) neurons, which activate anorexigenic pathways [67, 68] and agouti-related peptide (AGRP) and neuropeptide Y (NPY) neurons that transfer appetite stimulating signals. A decrease in leptin is correlated with an increased food intake [67].

By binding to LEPR in the hypothalamus, leptin causes Janus kinase 2 (JAK2) activation and LEPR tyrosine residues phosphorylation, allowing STAT3 to be dimerized and translocated to the nucleus, leading to anorectic peptide synthesis [67, 68]. Also, it has been shown that leptin's effects on food intake and body weight require inhibition of hypothalamic AMPK. Thus, hypothalamic AMPK plays a critical role in hormonal and nutrient-derived anorexigenic and orexigenic signals and in energy balance [69, 70].

3.3. Plasminogen Activator Inhibitor-1 (PAI-1). Plasminogen activator inhibitor-1 (PAI-1) is the most important endogenous inhibitor of fibrinolysis and increased levels are associated with insulin resistance, body weight control, and thrombosis. In humans, visceral adipose mass has been shown to be a primary determinant of PAI-1 levels. In T2DM, not only increased adipose tissue mass but other metabolic disturbances, including hyperinsulinemia, hyperglycemia, and dyslipidemia, alter adipose tissue function and lead to increased production and circulating levels of PAI-1 [25]. Consumption of fructose at 25% of energy requirements for 10 weeks leads to increases of fasting as well as postprandial PAI-1, suggesting the possibility that prolonged consumption of fructose may contribute to the development of metabolic syndrome via induction of prothrombotic (PAI-1) mediators besides proinflammatory cytokines [71].

4. Combined Signals

In the fed physiological state a multitude of gut hormones are released into the circulation at the same time depending on the quality and quantity of the diet with recommended proportions of the macronutrients as follows: carbohydrates 60%, proteins: 20%, and lipids: 20% (Figure 2). How the satiety factors act in concert to regulate appetite is still misunderstood.

Following the above-mentioned idea, after a high-protein meal, ghrelin declines gradually in both normal weight and obese children without subsequent increase, whereas ghrelin is suppressed more rapidly to a nadir at 60 min after a high-carbohydrate meal in both groups of children, followed by rebound in ghrelin levels. Similarly, after the high-protein meal, PYY concentrations increase steadily over the course of the morning in both groups without decline, whereas PYY levels peaked 30 min after the high-carbohydrate meal in both normal weight and obese subjects with significant

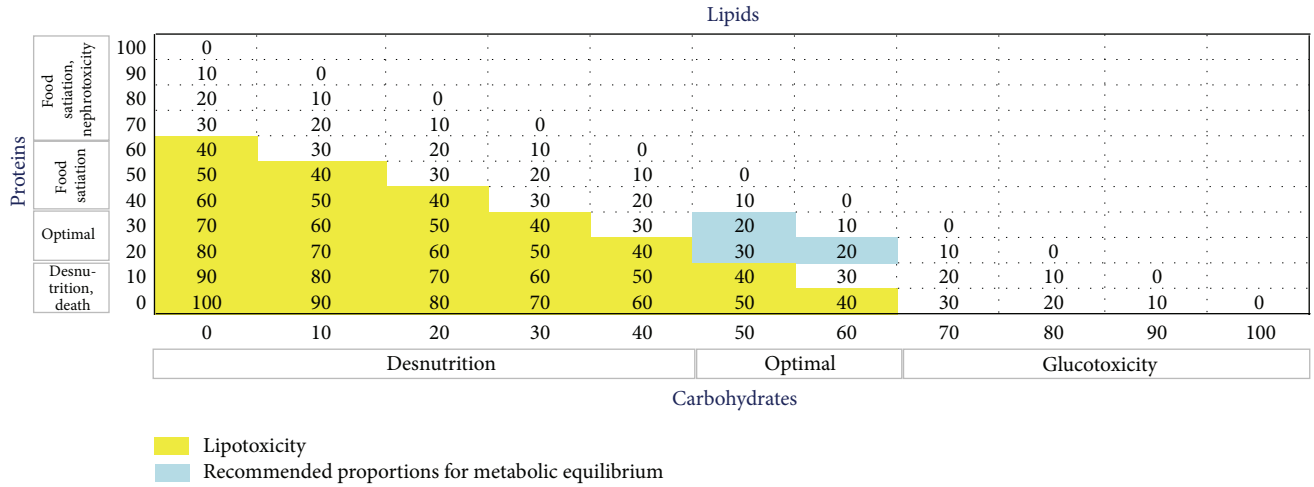

Figure 2: Recommended proportions of macronutrients.

decline thereafter. Ghrelin and PYY responses to the high-fat meal are somewhat intermediate between that observed with high carbohydrate and high protein [15].

Amylin, especially when combined with other anorectic hormones, has beneficial long-term effects on body weight. For example, amylin and GLP-1 mediate the feedback control of eating by seemingly separate but overlapping mechanisms. Another case is CCK, a synergic effect has been observed when applied simultaneously with amylin, estradiol, insulin, and leptin [72].

The combination of $PYY_{3\text{-}36}$ and GLP-17-36 amide produces a reduction in *ad libitum* energy intake in healthy, lean human subjects [73]. Recent work in investigating the utility of combinational therapies for the treatment of obesity has focused on the coadministration of amylin with leptin [74]. Moreover, combinational therapy of exendine-4 + sCT produced sustained daily food reductions without tolerance, nausea, malaise, or rebound feeding. These findings further support the view that engaging multiple feeding inhibitory pathways to reduce food intake could be a potential strategy for the treatment of obesity.

5. Peripheral Signals Modulated by Food

One strategy for the prevention of overweight and obesity related diseases is the use of agents that interfere with the hydrolysis and absorption of dietary carbohydrates and lipids. The most important dietary carbohydrates are starch, sucrose, and lactose. They are digested by disaccharidases in the upper gastrointestinal tract and broken down into monosaccharides. Subsequently they are absorbed to the circulation. The elevated glucose concentration in blood promotes insulin secretion from the β cells of the islets of Langerhans in the pancreas, and insulin mediates the uptake of glucose in peripheral tissues including muscle, adipose tissue, and kidney. Taking into account the importance of carbohydrate metabolism, the gastrointestinal enzymes can be therapeutic targets for limiting absorption of monosaccharides [75].

In addition, the most important dietary lipids are triglycerides and cholesterol esters. They are digested by pancreatic lipase and pancreatic phospholipase A2 to glycerol, fatty acids, and free cholesterol. Finally, they are absorbed to the circulation and may be used or stored in adipose tissue [76].

In the literature it can be found several reviews that describe active substances in plants that inhibit pancreatic enzymes. It has been recorded that more than 1200 plant species could have a hypoglycemic activity [77]. For example, Hanhineva et al. revised the inhibitory properties of polyphenols (i.e. flavonoids, phenolic acids, proanthocyanidins, and resveratrol). They reported that these polyphenols may influence carbohydrate metabolism at many levels. More interesting is that these compounds are contained in plant-based foods, such as tea, coffee, wine, cocoa, cereal grains, soy, fruits, and berries [78, 79].

Besides the food in their natural form, the heat processing of food (i.e., boiling) can produce derivate compounds that show digestive enzymes inhibitory properties. For example, it has been shown that after heat treating of raw ginseng, amino acid derivatives such as arginyl-fructose and arginyl-fructosyl-glucose are formed at high levels; these products inhibited postprandial hyperglycemia through the inhibition of α-amylase and α-glucosidase [80]. Other compounds such as flavonoids from grape seed Cat's whiskers and Sweetleaf extract obtained by heat processing also inhibited α-amylase [81].

It is worth mentioning that some plants do not show a significant effect in the inhibition of α-amylase; however, in combination with acarbose, an antidiabetic drug with α-glucosidase inhibitory properties has a synergistic effect due to low doses of acarbose that are necessary for the postprandial glycemic control. For example, the polyphenol extracts from a range of berries, especially raspberry and rowanberry, showed an effect only in combination with acarbose [82]. Other examples of this synergistic action are the inhibition by cyanidin-3-rutinoside [83] and some species of cinnamon [84].

The brush border enzymes are inhibited by molecules extracted from plants. For example, the D-fagomine from seeds of buckwheat inhibits sucrase [85], diacylated anthocyanin from purple sweet potatoes has been shown to inhibit maltase [86], cyanidin-3-galactoside inhibits sucrase and maltase [87], and α-glucosidase is inhibited by the hydromethanolic seed extract of *Holarrhena antidysenterica* [88], by ethanol extract of the fruit case of *Garcinia mangostan* [89], and by *Corni fructus* [90]. Flavonoids from grape seed Cat's whiskers and Sweetleaf extract inhibit intestinal sucrase and maltase [81] (which is also inhibited by some species of cinnamon) [84].

Enzymes from the metabolism of lipids can be also inhibited by compounds of the plants; for example, oligomeric procyanidins in the apple polyphenol extract inhibit pancreatic lipase [91], and arginyl-fructose and arginyl-fructosyl-glucose inhibit lipase [80]. Cocoa procyanidins inhibit pancreatic lipase, also pancreatic α-amylase, and phospholipase A2, and this inhibition produces a decrement in plasma triglyceride and glucose concentrations in mice as well as humans [92].

More studies are needed about the inhibitory activity of substances from natural origins (i.e., plants) on intestinal enzymes. Diabetic patients would beneficiate if they include these plants in their diet instead of active purified compounds. However, the concentrations in food of the active complexes could be not enough, then it is essential to get them in a purify form. For this reason, studies are needed in this area.

6. Evidence for the Existence of an Intestinal Missing Link

6.1. Gestational Diabetes Mellitus. Obesity increases the risks of gestational diabetes mellitus (GDM) [93, 94]. Even more, there seems to be a shared genetic basis between GDM and T2DM [95]. In fact, the diagnosis of GDM identifies patients with a pancreatic β-cell defect. In some patients, the defect is transient or stable, but in most it is progressive, imparting a high risk of diabetes for at least a decade after the index pregnancy.

The majority of women with GDM have clinical characteristics indicating a risk for T2DM. Available evidence indicates that T2DM can be prevented or delayed by intensive lifestyle modification and by medications, particularly those that ameliorate insulin resistance. All patients should be monitored for rising glycemia indicative of progressive β-cell deterioration. Monitoring should be initiated at least annually and should be intensified if glycemia is rising and/or impaired.

Like monitoring, lifestyle modification for obese and overweight women during pregnancy should be intensified for rising glycemia and/or development of impaired glucose levels. These measures restrict gestational weight gain and reduce the prevalence of gestational diabetes [96].

6.2. Obesity and Diabetes. Obesity, a BMI greater than 30 kg/m^2, is strongly and causally linked to T2DM. Recent data suggest that the prevention of diabetes is feasible if weight management is addressed. Modest weight loss of 5–10% body weight is known to improve diabetes by reducing insulin resistance in obese individuals [97]. Regarding this strategy, in clinical trials, caloric restriction, exercise, and weight loss have been shown to prevent and reduce diabetes in obese individuals [97, 98] in part by attenuating insulin resistance and subsequent hyperinsulinemia, thereby preserving β-cell function [99, 100].

While the goal of a cure for T2DM remains some way off, bariatric surgery has long been proven to be effective in weight reduction in the morbidly obese, as well as in maintaining long-term weight reduction. With this weight reduction, obesity-related comorbidities, including T2DM, tend to improve or resolve completely.

6.3. Bariatric Surgery. Bariatric surgery promotes effective and sustained weight loss in morbidly obese subjects [101]. Since 1991, several medical societies have established the criteria for bariatric surgery in cases with BMI > 40 or BMI > 35 with serious comorbidities [102].

Depending on the type of bariatric procedure, up to 80% resolution of T2DM has been reported, being more effective that those techniques that bypass the foregut like the Roux en-Y gastric bypass (RYGBP) [103–106]. Being more specific, Sugerman et al. found that a young age was a positive predictor for T2DM resolution [107] as well as early surgery that preempts irreversible pancreatic β-cell deterioration [108].

There are two different theories proposed to explain the laboratorial benefits after bariatric surgery. The hindgut theory by Cummings et al. [109] proposes that the rapid transit of nutrients to the hindgut improves glucose metabolism, probably through GLP-1. The second hypothesis, the foregut theory by Rubino [110], says that the exclusion of the foregut from the food stream causes a decrease in insulin resistance through the secretin pathway. The two theories are not mutually exclusive.

RYGBP causes an improvement in a diabetic patient's status through a variety of mechanisms. More interestingly, improvement often occurs very soon after the bypass, even before significant weight loss has occurred [111, 112]. First and foremost, RYGBP enforces severe calorie restriction through both mechanical restriction and the upregulation of satiety signals such as anorexigen PYY [113]. The decrease in caloric intake is by itself able to result in the improvement of T2DM [114].

One possible explanation for the metabolic improvement after RYGBP hypothesis is that bariatric surgeries with gastric bypass exclude the site responsible for the production of the hormone causing T2DM [115]. Other explanations are possible. For example, a hormone overproduced in the proximal foregut in diabetic patients might counteract the action of insulin, thus inducing insulin resistance and only secondarily hyperinsulinemia.

Collectively, evidence supports the concept that the effect of bariatric surgery on diabetes is mediated by a change in the pattern of secretion of gastrointestinal hormones [116],

supporting the use of them as therapeutic targets [117–119]. As a first instance, there is a greater insulin sensitivity due to a better β-cell function including the first phase of insulin secretion [120]. Also, there is restoration of a near-normal, postprandial insulin response soon after RYGBP [121], which is associated with a rise in GLP-1 levels [122]. Even more, ghrelin levels fall after RYGBP [109], resulting in appetite reduction.

It has been proposed that the bypass of the foregut in RYGBP restores normal GIP sensitivity and normalises the GIP levels [111], breaking the "GIP-resistant state," present in T2DM [123].

With the strong evidence published worldwide, surgery has been proven to be superior to medical treatment in terms of maintaining weight loss and altering the natural course of T2DM, which has been considered medically incurable [108, 124]. Despite the obvious risks of surgery [125], the risks of morbid obesity as well as all its associated comorbidities make surgery a viable option in those who are eligible.

7. The Adipose-Intestinal Missing Link

In susceptible individuals, chronic exaggerated stimulation of the proximal gut with fat and carbohydrates may induce overproduction of an unknown factor that causes impairment of incretin production and/or action, leading to insufficient or untimely production of insulin, so that glucose intolerance develops.

The bypass of the duodenum and jejunum might avoid a putative hormone overproduction in the proximal foregut in diabetic patients that might counteract the action of insulin, while the early presentation of undigested or incompletely digested food to the ileum may anticipate the production of hormones such as GLP1, further improving insulin action [126]. Moreover, GLP-1 has been implicated in the differentiation of pancreatic exocrine cells toward β cells by the Pdx-1 gene transcription stimulation. Indeed, GLP-1 increases the expression of β-cell-specific genes such as insulin, glucose transporter 2 (GLUT2), and glucokinase in human and rat pancreatic ductal cells transfected with Pdx-1 compared with those transfected with null vector [127].

Carbohydrates are mostly digested to glucose, fructose, and galactose before absorption by the small intestine. Absorption across the brush border and basolateral membranes of enterocytes is mediated by Na^+-dependent and -independent membrane proteins. Glucose and galactose transport across the brush border occur by a Na^+/glucose (galactose) cotransporter (SGLT1), whereas passive fructose transport is mediated by a uniporter (GLUT5). The passive exit of all three sugars out of the cell across the basolateral membrane occurs through two uniporters (GLUT2 and GLUT5). Mutations in SGLT1 cause a major defect in glucose and galactose absorption (glucose-galactose malabsorption), but mutations in GLUT2 do not appear to disrupt glucose and galactose absorption [128].

Because bariatric surgery with bypass obviates a great area of disaccharidases action, it is expected a reduction in glucose absorption which consequently leads to hyperglycemia improvement. Notwithstanding, a metabolic control would not be registered if there was not a β-cell recovery.

The common variable in the pathogenesis of GDM and T2DM is the weight gain surpassing recommended BMI. Furthermore, keeping a normal weight is fundamental in the prevention of these pathologies that are cured after a great weight loss coming in the puerperium or with bariatric surgery, respectively. This implies the role of circulating adipose signal acting on the proximal intestine that might inactivate a critical factor for the metabolic homeostasis (mainly insulin effect). SGLT1 or disaccharidases might be two target candidates to be affected by this adipose tissue derived factor.

Tumour necrosis factor α (TNF-α) expressed in high circulating levels in obesity is a proinflammatory cytokine implicated in the induction of insulin resistance [129]. There is also evidence of TNF-α effects on the β cell, which may further contribute to T2DM, although, as this cytokine is expressed in many other diseases without causing T2DM is low the probability to be by itself the adipose-intestinal link of T2DM.

8. Conclusions

The combined effects of macronutrients on the predominant gut hormone secretion are still poorly understood. Thus, from a therapeutic perspective, targeting the interaction of appetite signals in the gut offers the potential advantage of being able to manipulate appetite at a site distant from the CNS through endocrine and vagal nerve mechanisms [130].

Finally, future studies will target the identification of a proximal intestinal metabolic molecule, implicated as the cause or cure of T2DM whether activated or not.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 746281, 8 pages
http://dx.doi.org/10.1155/2013/746281

Review Article

Irisin, Two Years Later

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Received 28 June 2013; Accepted 1 October 2013

Academic Editor: Paolo de Girolamo

In January 2012, Boström and colleagues identified a new muscle tissue secreted peptide, which they named irisin, to highlight its role as a messenger that comes from skeletal muscle to other parts of the body. Irisin is a cleaved and secreted fragment of FNDC5 (also known as FRCP2 and PeP), a member of fibronectin type III repeat containing gene family. Major interest in this protein arose because of its great therapeutic potential in diabetes and perhaps also therapy for obesity. Here we review the most important aspects of irisin's action and discuss its involvement in energy and metabolic homeostasis and whether the beneficial effects of exercise in these disease states could be mediated by this protein. In addition the effects of irisin at the central nervous system (CNS) are highlighted. It is concluded that although current and upcoming research on irisin is very promising it is still necessary to deepen in several aspects in order to clarify its full potential as a meaningful drug target in human disease states.

1. Introduction

Obesity is at present the most common nutritional disease in industrialized countries, constituting a priority health problem. It is associated with the development of cardiovascular disease, *diabetes mellitus* type II, increased incidence of certain forms of cancer, and respiratory complications from other diseases, which leads to higher rates of mortality and morbidity, reducing directly or indirectly the quality and life expectancy of sufferers [1, 2]. Lifestyle modification, specifically changes in diet, physical activity, and exercise, currently continues to be the best option for treatment of obesity. In this sense, the benefits of exercise have been extensively documented [3]. Moreover, it has recently been reported that especially during or immediately after physical activity, skeletal muscle releases into circulation several hormones. These hormones, named myokines, can influence metabolism and modify cytokine production in different tissues and organs. On the basis of this, the concept of skeletal muscle must be reconsidered and being truly considered as an endocrine organ [4, 5].

Since human brown adipose tissue (BAT), especially in adults, was rediscovered several years ago by using positron emission tomography (PET) [6–9], it has been postulated as a major candidate for the treatment of obesity. This is based on the fact that brown adipose cells can dissipate energy in the form of heat leading to weight loss. This process takes place through a specialized mitochondrial protein called uncoupling protein 1 (UCP1). The uncoupling activity of UCP1 is explained by its ability to transport protons across the inner mitochondrial membrane, avoiding ATP synthesis and dissipating energy as heat [10]. Regulation of UCP1 is mainly at transcriptional level, where peroxisome proliferator-activated receptor Υ coactivator 1α (PGC1α) plays a key role [11].

Studies in immortal preadipocyte lines from the brown adipose tissue of mice lacking PGC1α corroborated their importance in thermogenesis [12]. Another important characteristic is its role in mitochondrial biogenesis; in fact the increased expression of PGC1α is parallel to increased mitochondrial DNA and gene expression of OXPHOS system (oxidative phosphorylation) in BAT [13, 14]. Although PGC1α

is mainly expressed in BAT, it is also expressed at higher levels in red, oxidative muscle. In fact, its expression is increased by exercise in mice, in rats, and in human beings [15]. Exercise rapidly and robustly increases the expression of PGC1α, but this effect is transient as both mRNA and protein levels of PGC1α quickly revert to preexercise values [16]. Exercise also activates AMP-activated protein kinase (AMPK), a master regulator of cellular metabolism. AMPK directly phosphorylates PGC1α, which is required for PGC1α-dependent induction of the PGC1α promoter [17]. While brief training produces only a transient rise in PGC1α, endurance training results in persistent PGC1α elevation [18]. Moreover, mice with transgenically increased PGC-1α in muscle showed improved metabolic responses as age related obesity and insulin insensitivity [19]. When the adipose tissue of these transgenic mice was analysed, it was observed that subcutaneous fat inguinal had significantly increased thermogenic gene program. These "brite" (brown-in-white) adipocytes display several classical brown adipocyte characteristics, as elevated levels of UCP1 mRNA and protein [20]. Further, other reports showed that exercise also enhances certain brown adipocyte-specific gene expression in the BAT, as well as white adipose tissue (WAT), suggesting that exercise training may induce important alteration in BAT and/or BAT-like phenotypic changes in WAT [21]. In this context it has been proposed that irisin, a recently discovered myokine, may be the molecule that links exercise with increased thermogenesis. In fact, irisin is named for Iris, the Greek goddess who served as courier among the Gods [20].

2. Irisin, A Bridge between Exercise and Thermogenesis

2.1. First Experimental Studies. Recently, Spielgman's group described that transgenic PGC-1α mice had greater levels of fibronectin type III domain containing (FNDC5) than wild-type mice [20]. FNDC5 (also known FRCP2 and PeP) is a type of transmembrane protein cloned by two groups in 2002. It has a signal peptide, two fibronectin domains and one hydrophobic domain inserted in the cell membrane [22, 23]. In fact, at present some authors question if FNDC5 might be a transmembrane receptor [24]. FNDC5 is proteolytically cleaved and secreted. Western blot of media fractions of cells overexpressing FNDC5 with antibodies against wild-type Fndc5 identified multiple bands; from 32 kDa to 20 kDa [20]. However, several aspects regarding proteolysis of this protein were not fully clarified yet. So, it seems that these possible discrepancies in molecular weight might be due to glycosylation in the culture media, while this is not observed in plasma mice. Moreover, the theoretically soluble secreted form, named irisin, would have a molecular weight of 12 kDa [20, 25] (Figure 1). A remarkable aspect about irisin is that the amino acid sequence is 100% identical among most mammalian species, which suggests a highly conserved function [20, 26].

Boström and colleagues demonstrated that irisin has potent effects on the browning of certain white adipose tissues, both in culture and *in vivo*. So, when they applied FNDC5 to primary subcutaneous white adipocytes during differentiation a great increase in oxygen consumption was observed which suggests higher energy expenditure. Moreover, the increase in uncoupled respiration was accompanied by an important induction of UCP1 mRNA and other known brown fat genes. However, genes characteristic to WAT were downregulated. Surprisingly, FNDC5 showed almost no effects on the classical brown fat cells isolated from the interscapular depot [20].

This evidence opened up some questions about the physiological role of irisin. In the same study, *in vivo*, it was demonstrated that injection intravenously of adenoviral vectors expressing full-length *Fndc5* resulted in *Ucp1* mRNA increased in the subcutaneous depot. Moreover, a moderate increase irisin blood levels caused a significant improvement in energy expenditure, body weight, and insulin resistance in mice that were fed a high fat diet. Finally, it was demonstrated that irisin was required for the effect of exercise in the browning of subcutaneous white fat and concluded that the rise in irisin is mediated by augmented concentrations of PGC1α in muscle, while PPAR-α (peroxisome proliferator-activated receptor-α) acts as downstream target of this hormone [20].

2.2. Interplay with Other Myokines. There is an extensive literature about different exercise-related signals that can regulate the expression and/or secretion of the diverse myokines [4, 5, 27, 28]. In this context, it has recently been published that there is a close interaction between irisin and myostatin. Myostatin, besides being a critical autocrine/paracrine inhibitor of skeletal muscle growth, has been shown to play an important role in metabolism [29]. In fact, it has been described as myostatin-knockout mice ($Mstn^{-/-}$) that show an increase muscle mass and a concomitant reduction of fat mass. Moreover, these mice show WAT with characteristics of BAT, an effect mediated by the AMPK-PGC1α-FNDC5 pathway in muscle [30].

Other studies have tried to elucidate the irisin role and other myokines in different physiological conditions. When male rats were subjected to calorie restriction (CR; ~60% *ad libitum*) there were no significant diet-related differences in plasma levels of myonectin, myostatin, or irisin, although there were significant changes in fat and lean mass, and also in insulin resistance [31]. These results may indicate that alterations in plasma concentration of these proteins are not essential for the CR-related improvement in insulin sensitivity in rats; however, it does not rule out that these plasma proteins may be relevant for some of caloric restriction's metabolic effects. On the other hand, Sánchez and collaborators have studied the possible effects of free fatty acids (FFA) alone and combined with adrenaline and AICAR (an activator of AMPK that acts as an exercise mimetic precursor) in the production of the myokines IL6, IL15, and irisin in mouse muscle cells *in vitro* [32]. They observed that FFA, adrenaline, and AICAR have a great influence in the IL6 expression and secretion, a little inhibitory effect on IL15 expression and almost no effect on the expression of FNDC5. In fact, the authors only found that FNDC5 had a tendency to be reduced with FFA and AICAR at isolated specific time

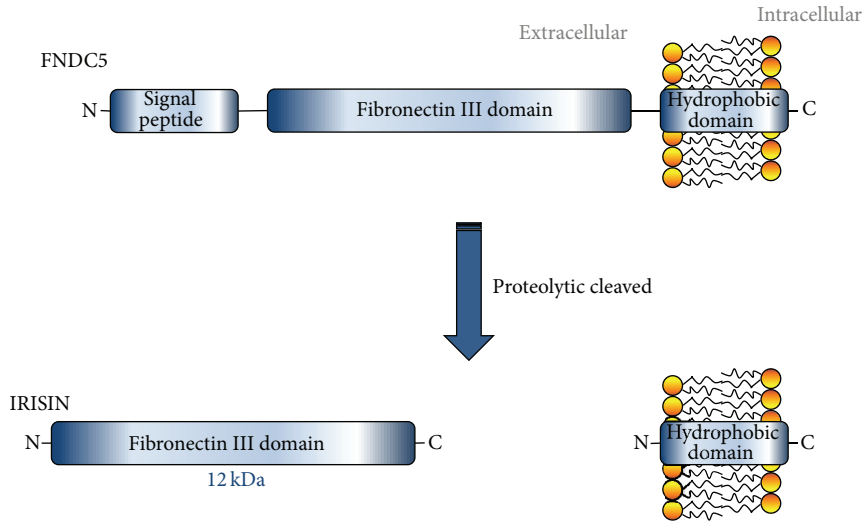

Figure 1: Expression of FNDC5 (fibronectin type III domain containing 5), also known as FRCP2 and PeP, is stimulated in muscle by PGC1-α in response to exercise. It is a signal peptide with two fibronectin domains in its amino (N)-terminal part and a hydrophobic domain inserted in the lipidic bilayer at carboxy (C)-terminal domain. The first 29 aa of the mouse FNDC5 are a signal peptide, followed immediately by the single FNIII domain of 94 aa. The next 28 aa are of unknown structure and function and contain the putative cleavage site for irisin. This is followed by a 19 aa transmembrane domain and a 39 aa cytoplasmic domain. FNDC5 is thus a type I transmembrane protein with its FNIII domain extracellular, similar to some cytokine receptors. This structure is synthesized as a type I membrane protein and followed by proteolytic cleavage realising amino (N)-terminal part of the protein into the extracellular to circulation.

points. Thus, it would be possible that more signals may be required *in vivo* for inducing FNDC5 expression. In this sense, recent evidence using human rhabdomyosarcoma cells showed that treatment for 24 and 48 hours with omega 3 fatty acids significantly induced irisin expression [33]. Finally, it has also been found that just as FNDC5, heart-derived natriuretic peptides activate white adipose thermogenic programs [34]. Taken together, these results may suggest that tissues such as skeletal and heart muscle, involved in high energy-expending activity, send signals to adipose tissue [35, 36].

2.3. Irisin Is Also an Adipokine. Current data by Roca-Rivada and coworkers proposed that irisin is not only secreted by muscle tissue. In fact, they demonstrated that irisin is a new adipokine with an important autocrine and endocrine function. Moreover, they showed that FNDC5/irisin has a different pattern of secretion depending on the anatomical location of adipose tissue. Thus, subcutaneous adipose tissue secretes more much FNDC5/irisin than visceral adipose tissue, reflecting one more time that visceral fat is more implicated in metabolic complications, while subcutaneous fat has a possible beneficial role. They also showed that short-term periods of exercise training induced FNDC5 secretion by WAT, that this secretion was significantly reduced in fasting animals, and that WAT of obese animals had an increase secretion of this hormone suggesting a type of resistance [25]. Another interesting feature, also reported by those authors, indicates that FNDC5/irisin has a secretion profile similar to other adipokines like leptin. Moreover, it is suggested that this hormone might be implicated in the regulation of circulating FNDC5/irisin levels. In fact, Zucker obese rats with no functional leptin receptor showed significantly diminished levels, while DIO (diet induce obesity) rats showed a significant increase. Ultimately, all these results suggest an interactions between muscle and adipose tissue interaction a regulatory feedback mechanism.

In this same context, Roberts et al. showed that obese/diabetic prone Otsuka Long-Evans Tokushima Fatty (OLETF) rats have more muscle expression FNDC5 than lean Long Evans Tokushima Otsuka (LETO); however, LETO rats have higher circulating irisin levels. The authors also observed that triceps FNDC5 mRNA expression was correlated with fat mass and with plasma leptin; however, *in vitro* leptin treatment had no effect on FNDC5 mRNA expression in myotubes [37]. Given that the effect of leptin treatment depends on endogenous levels of this hormone and on the physiological state [38], many studies are still needed to determinate a possible interaction between leptin and irisin in the so-called muscle-adipose tissue axis.

3. Irisin, Potential Roles in the Central Nervous System

Besides interaction between skeletal muscle and adipose tissue, it has been described that FNDC5/irisin might have a role in the central nervous system. In fact, it has already described previously that PGC1-α, an upstream of FNDC5, benefits tissues that do not have a primary metabolic function, such as the brain [39–41]. In this context, immunohistochemical studies have recently revealed that rat and mice cerebellar Purkinje cells expressed irisin and also FNDC5 [42]. Furthermore, the same authors hypothesize about a novel neural pathway, where irisin produced in cerebellum might regulate

adipocyte metabolism via several intermediary synapses in the medulla and spinal cord, an interesting idea that still requires to be confirmed.

Supporting the role of FNDC5/irisin in the nervous system, it should be noted another study where it is demonstrated that FNDC5 is required for the adequate neural differentiation of mouse embryonic stem cells (mESCs) [43]. The authors observed that both *Fndc5* knockdowns in mESCs during their differentiation after postneuronal progenitor formation and the neuronal differentiation were reduced. Finally, Moon et al. showed that hippocampal neurogenesis is regulated by irisin in a dose-dependent manner. So, while physiological concentrations of irisin (5–10 nmol/L) had no effect on mouse H19-7 hippocampal neuronal cells proliferation, pharmacological concentrations (50–100 nmol/L) increased proliferation when they were compared to control. This increase seems to occur trough signal transducer and activator of transcription (STAT)3 but not AMPK and/or extracellular signal-regulated kinase (ERK) signalling pathways [44].

Overall this evidence suggests a central role for irisin, In this regard, considering that the hippocampus is one of the principal regions affected by Alzheimer's disease and that exercise causes neurogenesis in humans reducing risk of Alzheimer's [45], Parkinson's, and some other neurodegenerative diseases [26, 46], irisin might be the link between exercise and healthy brain. Another interesting question that needs to be addressed is whether irisin may be expressed and play a role in other brain areas involved in the regulation of energy balance, such as the hypothalamus and the brainstem.

4. Irisin, Studies in Humans

4.1. Human Exercise Gene. As stated above, irisin has a highly conserved function, and as in rodents, in humans this hormone is also predominantly expressed in muscle [47]. While, available data indicates that this is the main source of production, it was also found that both subcutaneous and visceral adipose tissue expressed and secreted FNDC5/irisin [25, 48]. On other hand, circulating irisin was detected in the serum or plasma of all subjects studied, whereas circulating FNDC5 was detected in only a minority of the subjects [47], which could be explained by a different processing in a minority group of humans.

Throughout the past two years, several studies in humans have tried to clarify the role of FNDC5/irisin in physiological conditions and in disease states. Spielgman's group showed that endurance exercise training for 10 weeks in healthy adult humans increased plasma irisin levels compared to the baseline state [20]; however, there are some discrepancies about this. While Huh et al. also observed that circulating irisin levels were significantly upregulated 30 min after acute exercise [47], another study have questioned those results. So, other study has not been able to reproduce FNDC5 gene activation by aerobic exercise in younger subjects or in a resistance training study in 20–80 year olds [49]. These authors question therefore whether irisin is a human exercise gene. These discrepancies might be explained as that an increase in irisin levels occurs in states where more energy is needed, such as untrained individuals, while among trained individuals it is not necessary [47]. In the same direction, a recent study confirms that neither longer-term nor single exercise markedly increases skeletal muscle FNDC5 expression or serum irisin [50].

It seems, hence, that exercise might have an effect on irisin levels depending on physiological condition. In this sense, a new study describes that patients subjected to hemodialysis seem to have lower plasma irisin than healthy subjects and also show exercise training resistance; so despite increasing muscle mass, they not have higher irisin levels [51].

4.2. Metabolic Diseases. When analyzing the correlation between body max index (BMI) and irisin levels, differences were also found. Some studies observed a positive correlation with BMI [47, 52, 53], while other reported null [49] or even a negative correlation [48]. It would be necessary a deeper investigation in this field, and a possible resistance to this protein should be characterized, as animal studies suggest [25]. In addition, bariatric surgery-induced weight loss has been reported to decrease irisin levels, independently of BMI [47]. However the functional significance of this finding needs further exploring.

Similarly, it has been established by some groups a relation between *diabetes mellitus* type 2 (DMT2) and irisin levels, although it is also reported that irisin expression is not related to diabetes status in humans [49]. Most studies show lower irisin levels in patients with DMT2 [48, 54, 55]. Fernandez-Real's group suggests that a lesser production of irisin in muscle/adipose tissue in obese and patients with DMT2 could be responsible of the obesity-associated lower brown or beige adipocytes in human adipose tissue. So, they consider increasing irisin levels and browning adipose tissue as a potential target for metabolic diseases' treatment [48].

In this same context, another controversy has been reported. The study of single nucleotide polymorphisms (SNPs) in the human *Fndc5* locus, encoding the irisin precursor, showed that a common genetic variation in this locus determines insulin sensitivity [56]. Moreover, data from human myotubes revealed a negative association between FNDC5 expression and *in vivo* measures of insulin sensitivity. This result appears conflicting with the mouse data from Boström et al. who reported reduced insulin resistance in high fat-fed mice after adenoviral *Fndc5* overexpression [20]. Considering the association of DMT2 and cardiovascular disease, a role for irisin is also tempting to be speculated. In this sense the FNDC5 expression in a skeletal muscle biopsy from heart failure (HF) patients, it was observed that this expression relates to functional capacity in a human HF and that a decrease in FNDC5 expression might reduce aerobic performance in HF patients [57].

Circulating irisin has been also found to be directly associated with muscle mass and estradiol levels and inversely associated with age in middle-aged women. Also it is negatively correlated with age, insulin, cholesterol, and adiponectin levels [47, 52, 58], as well as intrahepatic triglyceride contents in obese adults [59]. While, another paper

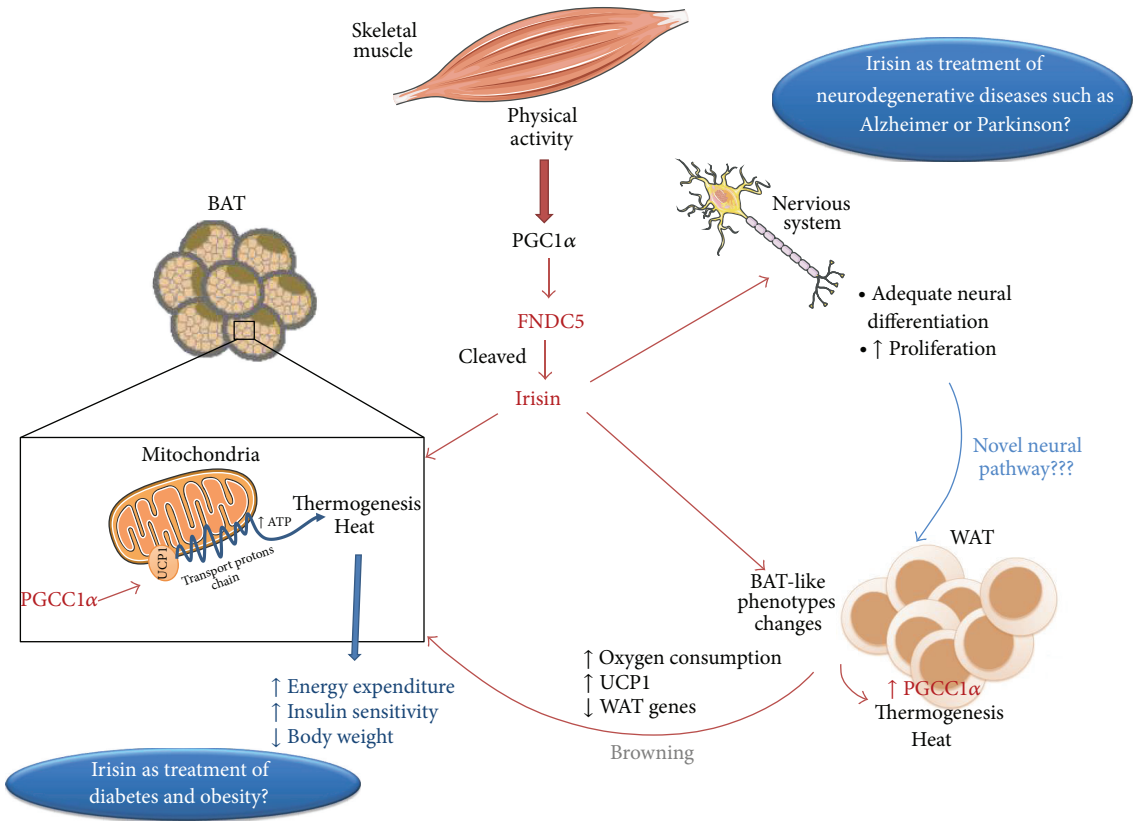

Figure 2: Skeletal muscle releases to circulation several hormones denomined myokines acting as endocrine organ. Thus during exercise PGC1α is activated inducing FNDC5 release which is cleaved to irisin. Irisin can act on different tissues, thereby brown adipose tissue activates UCP1 in mitochondria triggering transport protons chain in the mitochondrial membrane, resulting ATP increased and dissipating energy in form of heat. This process increases energy expenditure, reduces body weight, and improves metabolic parameters such as insulin sensitivity. Irisin on white adipose tissue stimulates BAT-like phenotypes changes, increasing PGC1α expression and thereby UCP1 and oxygen consumption while decreases WAT genes, process in which WAT stops behaving as energy reservoir for to use fat as energy source as in BAT, process named browning. For all of this, irisin has been proposed as a possible novel treatment in diabetes and obesity. Other target of irisin is nervous system where preliminary studies suggest that it could act on adipocyte metabolism through a novel neural pathway and on the other hand irisin induces neural proliferation and adequate neural differentiation, so it could also be a therapeutic target for neurodegenerative diseases such as Alzheimer or Parkinson.

suggests that in a population of postmenopausal women with BMI between 24 and 45, irisin levels do not correlate with 24 h energy expenditure (EE); however, for a subpopulation with EE greater than predicted, irisin levels and EE are highly correlative [60].

Similar to physical activity, drugs might also increase irisin levels and thus affect lipid metabolism and improve risk among dyslipidemic and/or obesity individuals. Given recent data, everything seems to indicate that between these drugs, statins could have an important role in this sense [61]. In this context, recently, Gouni-Berthold and collaborators have described that simvastatin, a hypolipidemic drug member of the statins, increases irisin concentrations both *in vivo* as *in vitro* [62]. Although it could be postulated that this increase could be beneficial, for example, by influencing adipose tissue metabolism and insulin resistance, it will be necessary to determine if irisin levels are result of myocyte damage or/and a mechanism of statin-induced cellular stress protection [62].

Another disease with altered energy expenditure and with high prevalence of metabolic imbalance and abnormal energy homeostasis is also chronic kidney disease (CKD). It was observed that patients with CKD have lower irisin levels at rest, independently of high-density lipoprotein cholesterol levels. The mechanism underlying the decrease in irisin in CKD is unknown, even though it seems that indoxyl sulphate, which is a protein-bound uremic toxin, decreases FNDC5 expression in skeletal muscle cells and irisin level in the cell culture medium [58]. Authors consider that these results show good evidence on how uremia may affect irisin levels. Although this study has some limitations, it is suggested that irisin may be a novel therapeutic agent for treating metabolic diseases in CKD patients.

5. Future Prospects

When Böstrom and colleagues described irisin, rapidly, it was seen its great therapeutic potential. Irisin was seen as

possible treatment for diabetes and perhaps also therapy for obesity. Moreover, it also was considered a possibility to treat patients with Alzheimer's, Parkinson's, and some other neurodegenerative diseases. However, new studies have started to question the initial expectations [24, 63]. So, while clear-cut data have been reported in rodents, the thermogenic effect of irisin in humans remains controversial, and it is not clear if exercise has an impact on irisin levels [49]. In fact, recently, Raschke and coworkers described that neither FNDC5 gene is activated by contraction in humans nor has effect on "brite" differentiation of human preadipocytes [63]; even they propose that irisin function for mice is lost in humans. Thus, it seems obvious that further studies are needed to elucidate, in depth, this field.

First more studies would be necessary to determinate what the precise role of different forms of FNDC5/irisin is and if there is a different mechanism of proteolysis as it already was suggested [47]. On the other hand, it is absolutely necessary to characterize the receptor and the signalling pathway, which will allow a better understanding of irisin function. Just as with other hormones it seems to be a tolerance or resistance mechanism to irisin [25, 60]. So, the factors that contribute to irisin tolerance and/or resistance also would be defined. Similarly more extensive studies, with different cohorts, assessing genetic variations in the irisin gene and its relationships to obesity and associated comorbidities across life span are eagerly awaited. Another important aspect that we need to consider is that human BAT is closely related to rodent beige fat, rather than classical BAT; so if we want to study the irisin effect in human BAT, a rodent model with beige fat would be necessary [26]. Intensive research efforts are needed to use BAT as a target organ for treatment of metabolic diseases.

In conclusion, although current and upcoming research on irisin is very promising and nowadays we already know so much about it (Figure 2), it is still necessary to deepen in several aspects in order to clarify its full potential as a meaningful drug target in human disease states.

Acknowledgments

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under Grant agreement no. 281854—the ObERStress European Research Council Project(ML) and no. 245009—the Neurofast project (ML and CD), and Xunta de Galicia (ML: 10PXIB208164PR and 2012-CP070), Instituto de Salud Carlos III (ISCIII) (ML: PI12/01814), MINECO were co-funded by the FEDER Program of EU (CD:BFU2011-29102). CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative of ISCIII. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 518046, 10 pages
http://dx.doi.org/10.1155/2013/518046

Review Article

Role of GnRH Neurons and Their Neuronal Afferents as Key Integrators between Food Intake Regulatory Signals and the Control of Reproduction

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Received 28 June 2013; Accepted 7 August 2013

Academic Editor: Carlos Dieguez

Reproductive function is regulated by a plethora of signals that integrate physiological and environmental information. Among others, metabolic factors are key components of this circuit since they inform about the propitious timing for reproduction depending on energy availability. This information is processed mainly at the hypothalamus that, in turn, modulates gonadotropin release from the pituitary and, thereby, gonadal activity. Metabolic hormones, such as leptin, insulin, and ghrelin, act as indicators of the energy status and convey this information to the reproductive axis regulating its activity. In this review, we will analyse the central mechanisms involved in the integration of this metabolic information and their contribution to the control of the reproductive function. Particular attention will be paid to summarize the participation of GnRH, Kiss1, NPY, and POMC neurons in this process and their possible interactions to contribute to the metabolic control of reproduction.

1. Introduction

From an evolutionary point of view, the integration of the circuits controlling metabolism and reproduction is essential for the survival and perpetuation of the species. Wasting of energy on reproduction, in situations of deficit in food availability, may threaten the survival of the individuals and their progeny. Reproduction is a costly process in terms of energy consumption. Thus, in times of famine or under certain pathological conditions, which cannot ensure the correct utilization of the metabolic resources (anorexia nervosa, obesity, diabetes, lipodystrophies, etc.), the body has to define priorities to preserve physiological processes essential for survival [1]. In this sense, key physiological activities as blood circulation or neural activity cannot be compromised, whereas others, such as locomotion, thermoregulation, or growth can be reduced in conditions of metabolic stress, as occurs in hibernating animals.

In the above situations, reproduction is totally dispensable or even incompatible with survival since, besides the high energetic cost required to maintain the fertility, pregnancy and nursing limit considerably the mobility for food seeking. In wildlife, animals are more dependent on seasonal fluctuations in food availability than their counterparts living under domestic conditions or humans. However, the increased prevalence in developed countries of metabolic pathologies (ranging from anorexia to obesity and metabolic syndrome) makes the study of the metabolic control of reproduction of special interest, in part due to economic reasons. In this sense, it is interesting that the increase in the incidence of metabolic disorders during the last decades coincides also with increased rates of infertility, although direct association between these two phenomena has not been demonstrated so far [2, 3]. Of note, besides the elevated cost derived from the treatment of illness associated with metabolic disorders such as diabetes, hypertension, and other cardiovascular problems, the cost of a successful treatment by means of *in vitro* fertilization ranges from 19,588 to 134,190€ [4].

Changes in energy stores produce long- and short-term fluctuations in hormonal (leptin, insulin, and ghrelin) as well as nutritional (glucose, lipids) signals that feedback mainly

to the CNS to regulate metabolism and fertility. However, although many of the neural circuits controlling energy homeostasis are well characterized, the elements conveying nutritional information to the reproductive axis are yet to be conclusively defined.

The reproductive axis comprises three major elements, the hypothalamus, the pituitary, and the gonads, which form the so-called HPG axis. Gonadotropin-releasing hormone (GnRH) neurons, located in the preoptic area (POA) of the hypothalamus, induce gonadotropin stimulation at the pituitary, which subsequently increases gonadal hormone secretion. The coordinated actions of these elements, together with other peripheral factors, allow the integration of endogenous and environmental information to ultimately produce gametes and modulate sex behaviour.

2. GnRH Neurons and the Metabolic Control of Reproduction

GnRH neurons are located in an excellent position to be considered as the best candidate to relay metabolic information to the downstream elements of the HPG axis since (i) GnRH neurons are the final output of the brain controlling the reproduction, (ii) GnRH neurons can sense peripheral signals because they send multiple projections to the *organum vasculosum of the lamina terminalis* (OVLT) and median eminence where the permeability of the blood-brain barrier is very high, and (iii) GnRH mRNA and the patterns of GnRH secretion change in situations of metabolic stress, suggesting that GnRH neurons are influenced by nutritional reserves.

However, there is a growing body of evidence demonstrating that GnRH neurons may not be targeted directly by the above metabolic factors. In support of this idea, recent analyses demonstrated that the signal transducer and activator of transcription 3 (STAT3) is not presented in GnRH neurons after leptin administration, and that GnRH neurons lack leptin receptor (LepR), assessed by single-cell PCR. Further proof for the absence of direct actions of leptin on GnRH neurons came from functional genomic studies involving selective ablation of LepR in GnRH neurons or in all forebrain neurons. These experiments demonstrated that elimination of leptin signalling in GnRH neurons does not have any impact on reproduction, whereas LepR ablation in all forebrain neurons impaired fertility [5]. Along with leptin, insulin is another potential candidate to relay metabolic information to the reproductive system. In fact, neuron-specific insulin receptor-knockout (NIRKO) mice exhibit hypogonadism of central origin [6]. However, the direct influence of insulin on GnRH neurons or even the presence of insulin receptors (IR) in this neuronal population has been extensively questioned. Recent analyses have confirmed that, although IR is expressed in GnRH neurons, the selective ablation of IR on this neuronal population, by breeding $IR^{fl/fl}$ mice onto a GnRH-Cre background, does not alter the normal timing of puberty or fertility [7]. These data suggest that the positive effect of insulin on reproduction is not mediated directly via GnRH neurons.

Contrary to leptin and insulin, ghrelin is a signal of energy deficiency. Compelling evidences have highlighted the negative effect of ghrelin at different levels of the reproductive axis [8]. In this sense, chronic administration of ghrelin to peripuberal rats inhibits gonadotropin release and mimics the delay on puberty onset produced by situations of energy deficit, such as chronic undernutrition [9], conditions in which endogenous ghrelin levels are expected to be elevated. Besides its central effects, it has been demonstrated that ghrelin can also modulate the reproductive function through its actions on the pituitary and gonads [8, 10]. Ghrelin receptor GHS-R1a mRNA is present in many areas of the brain. However, conclusive demonstration of the expression of this receptor in GnRH neurons remains elusive. Nonetheless, studies in rats of the inhibitory effects of ghrelin on GnRH pulsatility (as evidenced by an increase of GnRH interpulse intervals) demonstrated that this can be blockaded by an NPY Y5R antagonist [11]. This suggests that ghrelin effects on GnRH neuron are mediated, at least in part, by afferent neurons (probably NPY neurons).

The above lines of evidence indicate that, although GnRH neurons are ultimately affected by metabolic signals, some key peripheral indicators of the energy status do not target this information directly on GnRH neurons. Some of the potential candidate afferents responsible for transmitting metabolic information to GnRH neurons will be reviewed in the following sections.

3. Kiss1 Neurons and the Metabolic Control of Reproduction

Kisspeptins, encoded by the *Kiss1* gene, are peptides derived from a common precursor named kisspeptin-54, which was originally termed metastin by its ability to suppress melanoma metastasis [12]. These peptides share a common RF-amide carboxyl-terminal region, which is essential for the activation of its G-protein-coupled receptor, GPR54. Both *Kiss1* and *GPR54* genes have been phylogenetically well conserved, certifying the importance of this system [13]. In 2003, two independent studies uncovered the indispensable role of kisspeptins and its receptor for the normal timing of the maturational events controlling the reproductive function. These two reports identified that inactivating mutations of GPR54 causes hypogonadotropic hypogonadism, suggesting that Kisspeptin signaling plays a major role in the control of the reproductive axis [14, 15]. Since then, a large number of papers have confirmed and extended this initial observation, thus deepening into the molecular and physiological mechanisms involved in the control of the reproductive function by the Kiss1/GPR54 system.

In rodents, Kiss1 neurons are organized in two different populations neuroanatomically separated within the hypothalamus. One of these populations is located in the so-called rostral periventricular area of the third ventricle (RP3V), which comprises the anteroventral periventricular nucleus (AVPV), the rostral preoptic periventricular nucleus (rPVpo), and the caudal preoptic periventricular nucleus (cPVpo) [16]. Several studies have demonstrated that this population is activated by estradiol and sends projections to GnRH neurons in the POA suggesting that RP3V Kiss1

neurons are potentially involved in conveying the estrogen-positive feedback [17, 18]. The other population, located in the arcuate nucleus (Arc), shows diametrically opposite responses to estradiol, as Arc *Kiss1* expression is inhibited by estrogen. This circumstance, together with the fact that Arc Kiss1 neurons also project (albeit to a lesser extent) to GnRH neurons in the POA, suggests that this neuronal population could be involved in mediating estrogen-negative feedback [19, 20]. Of note, however, although this assumption has been accepted for years, recent data from electrophysiological recordings have failed to document electrical activation of Arc Kiss1 neurons after withdrawal of the inhibitory effect of gonadal steroids in mice [21].

Kiss1 neurons have been also proposed as mediators for relaying metabolic information to GnRH neurons [22]. There are compelling evidences supporting this hypothesis. Fasting, which reduces significantly LH secretion, is correlated with diminished *Kiss1* expression [23–25]. Chronic administration of kisspeptin partially rescues the delay on puberty onset caused by chronic undernutrition [26]. Anovulation and reduced levels of GnRH/LH during lactation, caused by the negative energy balance due to milk production, are associated with reduced levels of *Kiss1* mRNA in the Arc [27]. Infertile leptin-deficient mice (ob/ob) show low levels of *Kiss1* mRNA, and leptin injection recovers, in some extent, *Kiss1* expression and fertility [28]. Likewise, streptozotocin-induced diabetic rats, which are leaner and hypoleptinemic, display low levels of *Kiss1* mRNA in the hypothalamus. However, chronic injections of Kisspeptin-10 partially rescued the negative effects of insulin deficiency on reproductive parameters (LH and testosterone secretion and prostate and testis weights) [29]. All in all, the above data point out that conditions of negative energy balance and metabolic stress that suppress the function of the HPG axis are associated with a detectable inhibition of the hypothalamic Kiss1 system.

Despite this solid evidence, whether Kiss1 neurons are direct targets for metabolic factors is still under discussion. Although previous studies assumed that almost half of the arcuate Kiss1 neurons express LepR [28], recent data demonstrated that the ablation of this receptor on Kiss1 neurons or its restoration on LepR-deficient mice does not have a detectable impact on reproductive function [30, 31]. In fact, a recent paper suggested that only a low percentage of Kiss1 neurons expresses LepR, only after completion of puberty onset. These results suggest that leptin may not act directly on Kiss1 neurons to control the reproductive function. However, it is highly plausible that leptin could modulate the activity of Kiss1 neurons through afferent inputs. In this context, it has been demonstrated that leptin signalling in the ventral premmamillary nucleus (PMV) is essential for puberty and fertility in mice, and LepR-expressing cells in this nucleus send projections to Kiss1 neurons [30]. This arises the possibility that Kiss1 neurons could act as downstream mediators for leptin actions on the PMV [32]. In this sense, recent data demonstrated that targeted lesion of the PMV disrupts *Kiss1* and *GnRH* expression during the proestrus-to-estrus transition in rats [33].

On the other hand, recent analysis revealed that Kiss1 neurons express IR, suggesting that Kiss1 neurons could sense metabolic status by receiving information about insulin fluctuations in bloodstream, to regulate reproduction. In fact, IR ablation from Kiss1 neurons, by Cre-loxP strategy, caused a moderate delay of puberty onset and a reduction of LH secretion in both sexes. However, lack of IR on Kiss1 neurons did not affect fertility in adults [34]. These data suggest that insulin signalling in Kiss1 neurons exerts, to some extent, a positive influence for the initiation of the puberty, although in absence of this stimulus, compensatory mechanisms are activated to complete the maturation of the reproductive axis.

In addition, the orexigenic hormone, ghrelin, which may play a major role disrupting fertility in situations of energy insufficiency, has been demonstrated to modulate *Kiss1* expression [35, 36]. Of note, intravenous injection of ghrelin has been shown to produce a significant decrease in LH pulsatility and *Kiss1* expression in the median POA (an area that includes the RP3V) of adult rats. These data suggest that Kiss1 neurons could contribute to mediate the negative effects of ghrelin on reproduction. However, whether Kiss1 neurons are direct targets for ghrelin remains to be clarified.

The above lines of evidence situate Kiss1 neurons as an excellent candidate responsible for the integration of metabolism and reproduction. However, many of the responses to metabolic hormones such as leptin, insulin, and ghrelin could be mediated by afferent inputs to Kiss1 neurons rather than by direct actions on this neuronal population.

4. Proopiomelanocortin Neurons and the Metabolic Control of Reproduction

Proopiomelanocortin (POMC) is a precursor protein that can be cleaved at different sites to generate several peptides with different biological activity. Among others, α-MSH, β-Endorphin, and ACTH are neuropeptides derived from the POMC precursor. In the brain, POMC neurons are divided into two different populations. One of them is located in the nucleus of the solitary tract (NTS). This is a small population comprised by nearly 190 neurons scattered throughout the dorsomedial and medial parts of the NTS [37]. The function of this neuronal population is still not well known. A recent study suggested that this population could be involved in short-term feeding suppression. However, selective ablation of NTS POMC neurons does not reproduce the obese phenotype showed by the global POMC-null mice [38, 39]. In addition, it has been demonstrated that these neurons do not respond to leptin [37]. Such evidences suggest that POMC neurons located in the NTS may have a marginal contribution to the global control of the energy homeostasis exerted by the POMC system. Further analysis will be necessary to unveil the function of the NTS POMC neurons.

The other population is located in the Arc. Unlike NTS POMC neurons, the function and phenotype of Arc POMC neurons have been extensively studied. This neuronal population coexpresses multiple neuropeptides (CART, dynorphin, and VGF) and neurotransmitters (GABA, glutamate, acetylcholine) [40–43]. In addition, Arc POMC neurons express a wide range of receptors, such as LepR, IR, and NPY Y1-R, that confer the ability to sense peripheral and central signals

involved in the control of metabolic homeostasis. This feature makes these neurons essential for the maintenance of energy status. In fact, selective ablation of Arc POMC neurons mimics the phenotype of the global POMC-null mice, producing increased food intake and reduced energy expenditure, resulting ultimately in obesity [38]. These lines of evidences situate POMC neurons as a central node for sensing body energy reserves and thus as key elements to finely tune the mechanisms involved in the control of food intake and energy expenditure to keep energy homeostasis. Accordingly, POMC mRNA expression in the Arc is reduced in leptin-deficient ob/ob mice, and leptin administration rescues this expression to that found in control mice [44]. Also, LepR deletion from POMC-expressing neurons disrupts body weight homeostasis, synapsis plasticity and produces hyperleptinemia [45, 46]. In the same vein, although IR ablation in POMC neurons does not cause any impact on body weight or glucose regulation [47], probably due to compensatory mechanisms, double LepR and IR deletion on POMC neurons produce higher negative impact on metabolic parameters than that found in the LepR KO mice [48].

The above lines of evidence demonstrate that Arc POMC neurons are key elements in the control of body weight and metabolism. This observation makes also POMC neurons a good candidate to relay metabolic information to GnRH neurons. Admittedly, information about the role of POMC products in the control of the reproductive axis is scarce and, in some cases, controversial. Yet, there is growing evidence suggesting that POMC neurons do participate in conveying metabolic information to GnRH neurons. In this context, whereas early studies demonstrated that independent ablation of LepR or IR on POMC neurons does not disrupt fertility [45, 47], mutant mice lacking both receptors in POMC neurons show severe reproductive deficiencies [48]. Moreover, immunohistochemical analyses demonstrated that POMC neurons project and make synaptic contacts with GnRH perikarya and nerve terminals, suggesting direct actions of POMC-derived peptides on GnRH excitability [49, 50].

Overall, the above data suggest that arcuate POMC neurons could act as a mediator for leptin and insulin actions upon GnRH neurons. In this sense, Watanobe showed, using push-pull perfusion techniques, that leptin infusion in the POA and median eminence stimulates GnRH/LH release, and this effect is preceded by an increase in α-MSH secretion [51]. Early pharmacological studies revealed that α-MSH is able to elicit a robust increase in LH in different mammalian species [52–54], as well as to stimulate sexual receptivity and lordosis behaviour in female rats [55]. However, discrepancies about the effect of α-MSH on LH can be found in the literature. In this context, there are data showing diminished or unchanged LH levels after α-MSH administration in rats [56, 57]. From these studies, it is apparent that steroid environment and administration site could influence LH response to α-MSH [58]. In this sense, recent data demonstrated that melanocortin receptors 3 and 4 (MC3/4R) agonist, Melanotan II, increases GnRH pulse generator activity in goats and this effect can be attenuated by estradiol [59]. This finding also suggests that the central actions of α-MSH on reproduction are mediated probably via MC3R and MC4R.

Unlike the clear role of MC3R and MC4R on metabolism, evidenced by the fact that MC4R KO and to a lesser extent MC3R KO mice develop obvious metabolic disorders [60, 61], its role in reproduction remains uncertain. Data derived from MC4R mutants demonstrated that although MC4R-deficient mice are fertile, females are poor breeders exhibiting reduced ovulation rates [62]. Moreover, males display erectile dysfunction and disturbed copulatory behaviour [63]. In the same way, MC3R KO males are fertile though females display a certain degree of subfertility [61]. Overall, it seems that although both MC3R and MC4R are involved in the control of reproduction, the lack of one type of receptor could be partially compensated by the other, resulting in a milder phenotype. Accordingly, mice with functional blockade of both MC3R and MC4R signalling pathways, by overexpression of its endogenous antagonist, Agouti-related peptide (AgRP), are infertile [64].

However, whether GnRH neurons are direct targets of melanocortin actions remained unknown until very recently, when electrophysiological recordings of GnRH neurons demonstrated that α-MSH increases cell firing in most of GnRH neurons through postsynaptic activation of both MC3R and MC4R [65]. Almost simultaneously, Israel et al. confirmed by single-cell RT-PCR that GnRH neurons express MC4R and showed that restoration of melanocortin signalling in leptin-deficient db/db mice recovers the normal timing of puberty onset and fertility, suggesting that melanocortin signalling is essential for leptin actions on GnRH neurons [66]. In good agreement, our preliminary studies have documented that the positive effects of leptin on puberty onset, in rats subjected to 20% daily caloric restriction, can be blocked to a large extent by the MC3/4R antagonist SHU9119 (manuscript in preparation). Overall, it seems that POMC neurons convey leptin actions on GnRH neurons directly through MC3/4R pathways. In any case, the existence also of indirect intermediaries between POMC and GnRH neurons cannot be discarded.

Although β-Endorphin is also derived from the POMC precursor, lines of evidence in the literature attribute to these neuropeptide effects diametrically opposite, in terms of metabolic and reproductive control, to those found for other POMC-derived peptides (such as α-MSH, γ-MSH, and ACTH). β-Endorphin mediates its actions mostly via μ-opioid receptor, although it displays also relatively high affinity by δ-, and κ-subtypes of opioid receptors [67]. Pharmacological experiments indicated that β-Endorphin increases food intake and body weight gain, whereas the opioid receptor antagonist, naloxone, inhibits feeding behaviour [68, 69]. Albeit there is consensus in the pharmacological studies about the stimulatory actions of β-Endorphin on food intake, paradoxically, mutant male mice retaining all the POMC-derived peptides except β-Endorphin show increased food consumption and are obese [70].

So far, pharmacological studies have shown mainly inhibitory actions for β-Endorphin on reproduction. Several analyses revealed that β-Endorphin inhibits basal GnRH/gonadotropin secretion in different species and physiological conditions [71–75], as well as the electrical activity of a subpopulation of murine GnRH neurons [65]. Also, central

injection of β-Endorphin is able to block the preovulatory surge of LH [76] and to inhibit sexual behaviour [77]. Accordingly, naloxone administration consistently increases LH release [78–80]. Interestingly, naloxone administration to amenorrhoeic women is able to elicit a potent LH response, suggesting that excessive opioid activity could be involved in some pathophysiological conditions resulting in amenorrhea [81]. Intriguingly, mutant mice lacking β-Endorphin display a normal reproductive phenotype, showing normal puberty onset and fertility [82], which could be attributed to compensatory mechanisms mediated by other opioids.

The fact that β-Endorphin is able to negatively modulate GnRH/gonadotropin release opens up the possibility that POMC neurons could integrate also negative inputs to suppress GnRH activity via this opioid. In this sense, recent data suggested that the signal of energy insufficiency, ghrelin, suppresses the reproductive axis via β-Endorphin. Thus, Ogata et al. showed recently that central administration of ghrelin reduces significantly LH concentration and pulse frequency, whereas naloxone is able to block this effect [83]. However, whether β-Endorphin acts directly on GnRH neurons or via other intermediate neurons is still under discussion. In this context, while electrophysiological recordings in guinea pig demonstrated that the μ-opioid receptor agonist, DAMGO, inhibits GnRH neurons postsynaptically [84], data from the teleost fish, medaka, indicated that β-Endorphin reduces action potential firing in GnRH neurons via indirect mechanisms [85]. On the other hand, there is a large number of data demonstrating that rat GnRH neurons do not express opioid receptors [86, 87]. Admittedly, part of the above discrepancies could be attributed to interspecies differences. Regarding the possible indirect effects of β-Endorphin on GnRH neurons, pharmacological analyses suggested that β-Endorphin modulates GnRH release via glutamate-nitric oxide pathway [88].

CART-immunoreactive neurons have been identified in many different hypothalamic (paraventricular, arcuate, dorsomedial, and ventral premmamillary nuclei, as well as lateral hypothalamic area) and extrahypothalamic nuclei (central amygdala) [89–91]. However, the Arc POMC/CART population has received special attention due to its potential role in regulating food intake and reproduction, while little is known about the intervention of other CART populations in the integration of these two systems.

Central administration of CART dramatically suppresses food intake [92, 93]. However, the receptor responsible for CART actions has not been identified yet. Unlike other Arc POMC neuropeptides, evidence for CART actions on the reproductive axis remains rather scarce. Nonetheless, data from *in vitro* incubation of hypothalamic explants showed that both CART and leptin are able to stimulate GnRH secretion by reducing interpulse intervals. Interestingly, coadministration of an anti-CART antiserum completely blocked CART effects on GnRH pulsatility whereas partially abrogated leptin actions [94, 95]. This means that Arc POMC neurons can mediate leptin actions on GnRH neurons, at least partially, through CART secretion. Intriguingly, very recent analysis demonstrated that CART could stimulate GnRH excitability both postsynaptically and presynaptically. The latter, probably via indirect actions through Kiss1 neurons [96].

Altogether, the above lines of evidence suggest that POMC neurons are key elements for conveying a large variety of metabolic inputs, ranging from signals of nutrient deficiency to energy sufficiency cues, to control the reproductive axis by secreting a wide diversity of neuropeptides with different, in many cases opposite, actions.

5. NPY Neurons and the Metabolic Control of Reproduction

NPY is member of a family of peptides that include also peptide YY (PYY) and the pancreatic polypeptide (PP). In rats, NPY-expressing neurons are widespread through different areas across the brain including, among others, the olfactory bulb, striatum, hypothalamus, spinal cord, and pineal gland, being the Arc and the paraventricular nucleus, within the hypothalamus, two of the nuclei containing higher concentrations of NPY neurons and fibers [97]. The Arc population coexpresses also AgRP and has been postulated as key element in the control of feeding behaviour [98]. In fact, in 2011 Aponte and co-workers demonstrated in an extremely elegant study that activation of arcuate NPY/AgRP neurons, using optogenetic tools, produces rapid stimulation of seeking behaviour and compulsive feeding in mice [99]. Interestingly, although AgRP is the endogenous antagonist of MC3/4R, this effect was demonstrated to be independent of melanocortin signalling suppression. These evidences suggest that Arc NPY neurons are essential to stimulate food consumption in situations of negative energy balance.

So far, five different NPY receptors (Y1, Y2, Y4, Y5, and Y6) have been identified, which show different affinity for the various peptides of the NPY family [100–103]. These receptors present different distribution patterns within the brain and participate in the regulation of multiple functions [104, 105].

Regarding the potential role of NPY in reproduction, Arc NPY neurons send projections to GnRH perikarya and nerve terminals and have been suggested to participate in conveying information about energy insufficiency to the reproductive axis [106]. In fact, food restriction markedly increases NPY mRNA, and this is well correlated with reduced LH release [107]. In addition, the infertile phenotype displayed by ob/ob mice has been associated with high levels of NPY mRNA. Indeed, deficiency of either NPY or its Y1 or Y4 receptors in these animals rescues fertility [105]. Despite these compelling lines of evidence, the regulation of the gonadotropic axis by NPY is rather complex. In fact, pharmacological studies showed opposite effects of NPY on LH release depending on the steroid milieu and receptor agonist used. Thus, whereas NPY inhibited LH release in intact and castrated animals [108, 109], this neuropeptide induced opposite stimulatory effects on steroid-primed ovariectomized rats [110]. Interestingly, NPY KO mice do not display any reproductive alterations under normal conditions. However, fasting does not induce the expected decay in LH levels in these animals, suggesting that NPY signalling is needed for transmitting metabolic information when the conditions for reproduction are unfavourable due to reduced energy availability [111].

A recent electrophysiological study evaluated the role of NPY receptors on GnRH neuronal activity by subtractive

analysis. To address such a wide spectrum of potential effects, a selection of NPY receptor agonists was used to determine the possible influence of each individual receptor on GnRH activity. This procedure allowed to identify that Y1R activation inhibits murine GnRH neurons [65]. In good agreement, previous data showed that Y1R activation significantly decreases the number of calcium transients in GnRH neurons from nasal explants [112]. Interestingly, the same inhibitory effect was also described in rats through Y5 receptor [113]. So, differences between species may exist for the type of receptor that mediates the inhibitory actions of NPY on GnRH neurons. On the other hand, Y4 receptor activation by different agonists resulted in a potent postsynaptic stimulation of GnRH neurons. Overall, the former discrepancies about the dual inhibitory/stimulatory effect of NPY on LH secretion could be due to differences in the ratio Y1R/Y4R in the model evaluated. Further analysis will be necessary to clarify this phenomenon.

As it was mentioned before, Arc NPY neurons also co-express AgRP. Both neuropeptides show similar response to fluctuations in energy availability. In this sense, AgRP mRNA is also increased in fasting conditions in order to stimulate food intake [114]. However, the mechanisms through which NPY and AgRP influence feeding behaviour are different. Accordingly, AgRP increases food intake, at least partially, by blocking the anorexigenic effect of endogenous MC3/4R activation by melanocortins [115].

Taking in account the clear stimulatory effect exerted by melanocortins on reproduction, opposite results might be expected for AgRP. In fact, early analysis showed that AgRP suppresses LH pulsatility in ovariectomized monkeys [116]. In addition, AgRP ablation in ob/ob mice rescued fertility [117]. Moreover, electrical recordings in GnRH neurons demonstrated that AgRP administration prevents the excitatory effect of the MC3/4R agonist, Melanotan II [66]. However, later results revealed that AgRP exhibits also stimulatory effects in a small subpopulation of GnRH neurons [65]. This paradoxical effect could be mediated via a mechanism independent of MCR3/4 receptors, a possibility that had been already considered in the context of other studies, which showed AgRP actions unexplained via MCR3/4 blockade [118, 119].

Overall, the above data suggest that Arc NPY neurons are able to module reproductive function through different mechanism involving NPY secretion and/or inhibition of melanocortin signalling by AgRP in situations of energy deficiency .

6. Conclusions

Reproductive function is highly dependent on nutrient availability. To ensure an efficient utilization of the energy stores, redundant pathways are necessary. These circuits must detect situations of metabolic stress as to be able to derive energy resources to maintain essential physiological functions, while partially or totally suppressing reproduction until more favourable conditions are achieved. Of note, data in the literature suggest that many of metabolic signals informing the reproductive brain do no act directly on GnRH neurons, the final output in the brain controlling reproduction. Thus, afferent inputs are necessary to transmit metabolic information to GnRH neurons. In fact, in recent years, the existence of different neuronal populations that are able to sense peripheral and central indicators of the energy status to convey this information to GnRH neurons has been exposed by a large number of experimental studies. As reviewed herein, Arc POMC, NPY, and Kiss1 neurons have been proposed as key intermediary elements to carry out this function. Interestingly, there are solid lines of evidence suggesting that these neurons make direct contacts and are able to modulate the activity of each other [96, 120, 121]. This raises the possibility of the existence of a complex network of interconnected neurons that is involved in the precise sensing of the metabolic status and in the transmission of this information to GnRH neurons to consequently modulate the reproductive function. On the basis of the evidence summarized here, it is tenable to postulate that Kiss1, NPY, and POMC neurons are prominent elements of such a complex neuronal network. Admittedly, however, although for sake of concision this review has focussed only in this selected group of neuronal populations, it is likely that other partners exist on such circuitry responsible for central metabolic-reproductive interactions, such as neurons expressing galanin-like peptide (GALP), melanin-concentrating hormone (MCH), orexins, or corticotropin-releasing hormone (CRF) [122, 123]. While the evidence so far available suggests that the roles of the latter neuropeptides in the control of the HPG axis are less prominent, it remains a challenge for the future to decipher how major and subordinate metabolic regulators interplay with and impinge on the central elements of the reproductive axis.

Acknowledgments

This work is supported by the Marie Curie International Outgoing Fellowships (IOF) of 7th Framework Programme of the European Union. The author would like to thank the members of the Physiology Section of the University of Cordoba for their intervention in the generation of some of the results showed in this review and especially Dr. Tena-Sempere for his inspiring discussion of the results reviewed here and his valuable support and help for the preparation of this paper.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 483145, 21 pages
http://dx.doi.org/10.1155/2013/483145

Review Article

The Role of "Mixed" Orexigenic and Anorexigenic Signals and Autoantibodies Reacting with Appetite-Regulating Neuropeptides and Peptides of the Adipose Tissue-Gut-Brain Axis: Relevance to Food Intake and Nutritional Status in Patients with Anorexia Nervosa and Bulimia Nervosa

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Received 28 March 2013; Accepted 11 July 2013

Academic Editor: Carlos Dieguez

Eating disorders such as anorexia (AN) and bulimia nervosa (BN) are characterized by abnormal eating behavior. The essential aspect of AN is that the individual refuses to maintain a minimal normal body weight. The main features of BN are binge eating and inappropriate compensatory methods to prevent weight gain. The gut-brain-adipose tissue (AT) peptides and neutralizing autoantibodies play an important role in the regulation of eating behavior and growth hormone release. The mechanisms for controlling food intake involve an interplay between gut, brain, and AT. Parasympathetic, sympathetic, and serotoninergic systems are required for communication between brain satiety centre, gut, and AT. These neuronal circuits include neuropeptides ghrelin, neuropeptide Y (NPY), peptide YY (PYY), cholecystokinin (CCK), leptin, putative anorexigen obestatin, monoamines dopamine, norepinephrine (NE), serotonin, and neutralizing autoantibodies. This extensive and detailed report reviews data that demonstrate that hunger-satiety signals play an important role in the pathogenesis of eating disorders. Neuroendocrine dysregulations of the AT-gut-brain axis peptides and neutralizing autoantibodies may result in AN and BN. The circulating autoantibodies can be purified and used as pharmacological tools in AN and BN. Further research is required to investigate the orexigenic/anorexigenic synthetic analogs and monoclonal antibodies for potential treatment of eating disorders in clinical practice.

1. Introduction

Anorexia nervosa (AN) and bulimia nervosa (BN) are eating disorders characterized by loss of self-control in eating behavior and disturbed emotions including high anxiety. These disorders affect 2-3% of young women [1]. AN is a serious eating disorder with the highest mortality rate among other psychiatric disorders [2, 3]. AN is characterized by chronic self-starvation, amenorrhea, and severe weight loss due to reduction of both fat mass and fat-free mass mainly at the expense of adipose tissue (AT). BN is an eating disorder in which the subject engages in recurrent binge eating. To compensate for the intake of the food and prevent weight gain, this is followed by induction of vomiting, use of laxatives, enemas, diuretics, excessive exercising, or fasting; this results in dysregulation of endogenous endocrine axes. In BN, the phenomenon of binge eating, that is, consumption of large amounts of food in a short time period, is accompanied by a sensation of losing control over eating [4].

The gastrointestinal tract, central nervous system, and AT referred to as the AT-gut-brain axis produce a series of hormones with orexigenic and anorexigenic effects [5–14] (Figure 1). On the one hand, ghrelin could represent a regulatory circuit controlling appetite and energy homeostasis by stimulating the release of other orexigenic peptides and neurotransmitters as well as neuropeptide Y (NPY) [15]. On

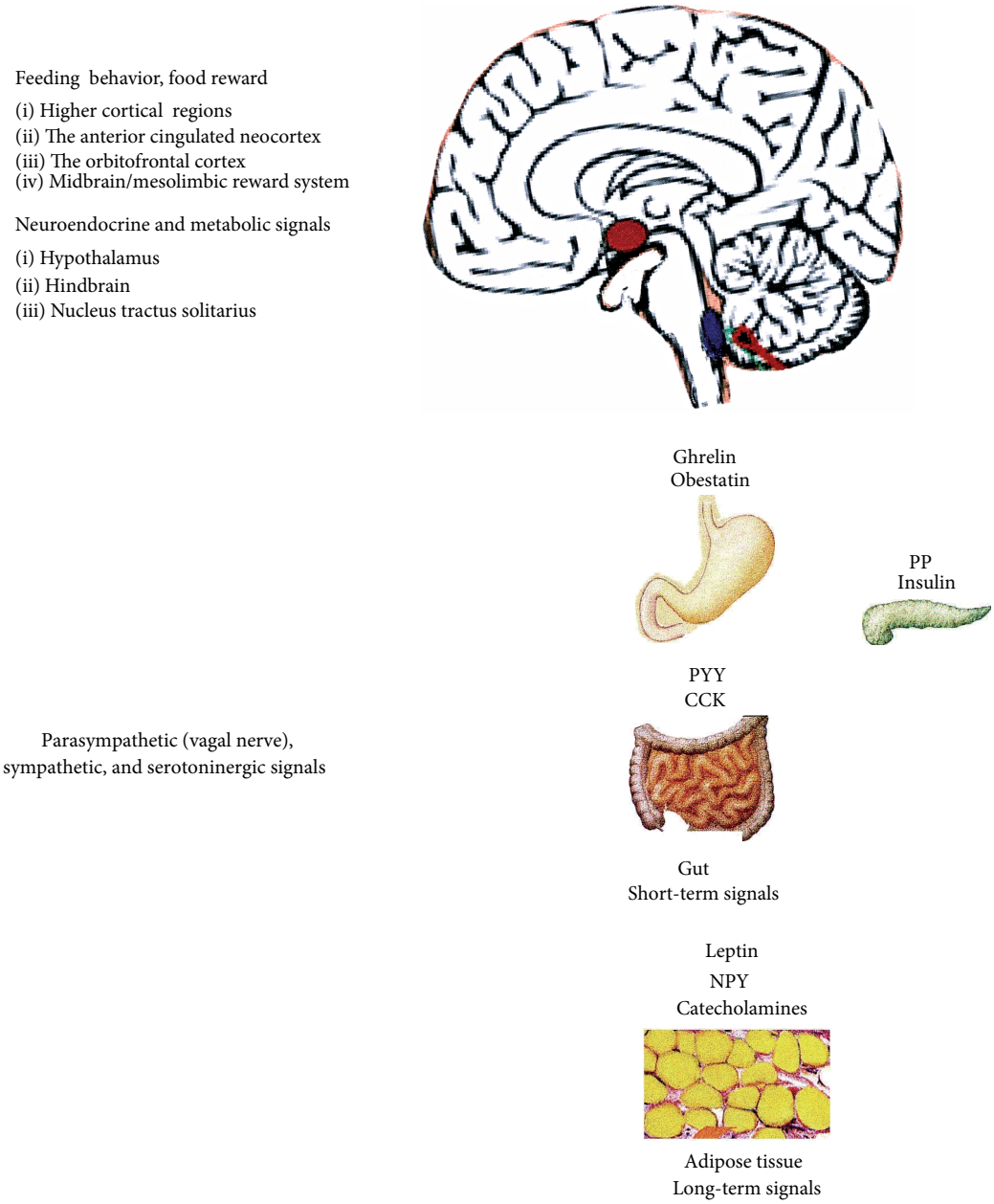

Figure 1: The role of adipose tissue- (AT-) gut-brain axis peptides in long-term and short-term regulation of food intake. Long-term regulators are adipose-derived food intake-inhibiting hormone leptin or food intake-stimulating hormone neuropeptide Y (NPY) mainly produced by the hypothalamus and also cosecreted with synthesized catecholamines in AT. Hormones produced in the gut are short-term food intake-stimulating hormone ghrelin, or food intake-inhibiting peptide YY (PYY), pancreatic polypeptide (PP), cholecystokinin (CCK), insulin, and putative anorexigen obestatin (the hypothalamus (violet), nucleus tractus solitarius (NTS, blue), sympathetic and serotoninergic areas (red), and vagal nerve parasympathetic area (green)).

the other hand, anorexigenic cholecystokinin (CCK), peptide YY (PYY), leptin, and putative anorexigenic hormone obestatin have an opposite effect at the hypothalamic level [16, 17]. The differential release of these hormones may act to initiate, maintain, or exacerbate cycles of food restriction or binge-purge behavior observed in AN and BN [18] (Tables 1 and 2). The former observations and recent reviews have suggested that AN and BN are linked to disturbed dopamine and serotonin systems [3, 19–23] which are related to anxiety, mood, and impulse control in patients with AN and BN. The inverse relationship between brain serotonin system and plasma ghrelin levels, hypothalamic NPY, and cocaine-amphetamine-regulated transcript (CART) expression in the regulation of feeding behavior was described in mice [24].

The abnormal eating behavior resulting in weight loss or weight gain contributes to an imbalance of energy metabolism and *in vivo* altered lipolysis and lipogenesis. Bradley et al. [25] hypothesized that orexigenic (appetite stimulating)

Table 1: Summary of the most relevant changes of adipose tissue-gut-brain axis plasma peptides stimulating appetite and autoantibodies against acylated ghrelin before and after refeeding in patients with anorexia nervosa (AN) and bulimia nervosa (BN), and the presence of autoantibodies against neuropeptide Y (NPY) and ghrelin in healthy women and autoantibodies against NPY in depressive disorder. Immunoglobulin (Ig) M, IgG, and IgA classes.

Peptides stimulating hunger and food intake and autoantibodies against peptides and autoantibodies against neurotransmitters	AN		BN	
	Acute phase	Weight restored	Acute phase	Recovered
NPY	→↑↓	→↓	↑→	→↑
NPY (response to test meal)	blunted		↓/blunted	
NPY (response to the exercise)			↑	
Autoantibodies against NPY in healthy women (IgG, IgA)				
Autoantibodies against NPY in depressive disorder (IgG↓)				
Ghrelin	↑	↑→	→↑	→
Ghrelin (response to test meal)	↓/blunted		↓/blunted	
Ghrelin (response to the exercise)			↓	
Autoantibodies against ghrelin in healthy women (IgG, IgA)				
Autoantibodies against acylated ghrelin (IgM)	↓	↑		
Autoantibodies against dopamine, dopamine-beta-hydroxylase, and serotonin (IgG, IgM)			↓	

↑: higher than healthy controls, ↓: lower than healthy controls, and →: not different from healthy controls.

Table 2: Summary of the most relevant changes of adipose tissue-gut-brain axis plasma peptides inhibiting appetite in patients with anorexia nervosa (AN) and bulimia nervosa (BN), and the presence of autoantibodies against leptin and peptide YY (PYY) in healthy women. Cholecystokinin (CCK), immunoglobulin (Ig) G, and IgA classes.

Peptides inhibiting hunger and food intake and autoantibodies against peptides	AN		BN	
	Acute phase	Weight restored	Acute phase	Recovered
Leptin	↓	→	→↓	→
Leptin (response to test meal)	→		→	
Leptin (response to the exercise)	↓		↓	
Autoantibodies against leptin in healthy women (IgG, IgA)				
CCK	↑→	→	↓→	↓→
CCK (response to test meal)	↑→	→	↓/blunted	
$PYY_{3–36}$	→↑↓		→	↑→
$PYY_{3–36}$ (response to test meal)	↑/blunted	→	↓/blunted/↑	
Autoantibodies against PYY in healthy women (IgG, IgA)				
Obestatin	↑→	↓	→↑	
Obestatin (response to test meal)	↓→		↓→	

↑: higher than healthy controls, ↓: lower than healthy controls, and →: not different from healthy controls.

neuropeptides promote positive energy balance and may potentially have antilipolytic properties, whereas anorexigenic (appetite-suppressing) neuropeptides promote weight loss and may stimulate lipolysis. The role of neuropeptides in mediating lipolysis and lipogenesis in humans is not well understood. Furthermore, sympathetic nervous system (SNS) and its neurotransmitter norepinephrine (NE) play a major role in regulation of AT lipolysis, appetite, energy expenditure, and the secretion of adipocytokines [26–30]. Very recently, it was revealed that the sympathetic innervation of AT is not only a source of catecholamines because adipocytes have the capacity to produce both NE and epinephrine [31] and that various stressors are able to stimulate production of catecholamines in adipocytes [32].

In our previous studies, we observed *in vivo* increased SNS activity in subcutaneous abdominal AT in AN and BN patients [6, 33–36]. The cause and pathogenesis of AN and BN, however, remain unknown. The existence of a complex neurotransmitter-neuropeptide pathology in AN and BN could explain the pathogenesis of individuals with the eating disorder [21–23, 37–42]. From the point of view of etiopathogenesis of AN and BN, it would be of interest to study the autoantibodies that react with neuropeptides and neurotransmitters which play an important role in eating

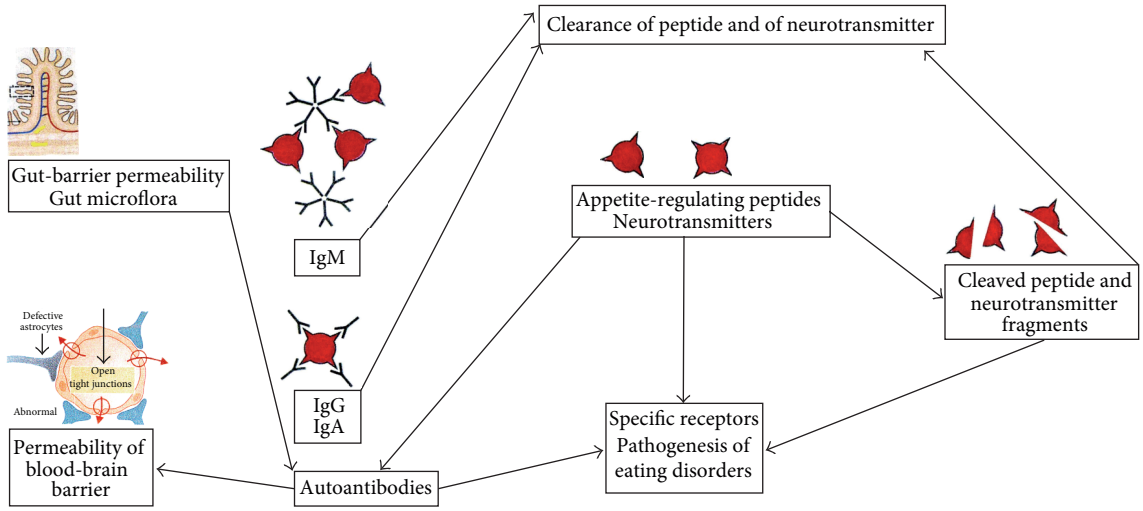

Figure 2: The role of up- or downregulated neutralizing autoantibodies (immunoglobulin (Ig) M, IgG, and IgA classes, and changes of their affinity) directed against appetite-regulating neuropeptides and peptides and neurotransmitters (dopamine, dopamine-beta-hydroxylase, and serotonin) in neuropeptidergic transmission and the pathogenesis of eating disorders. Producing excess of free fatty acids (FFA) and ketones to increase the permeability of the blood-brain barrier and to enter the cerebral matter in AN and BN [103]. Starvation, stress, catecholamines, microbial antigens, poststreptococcal autoimmune process (PANDAS), and proinflammatory cytokines decrease blood-brain barrier integrity in parallel with decreased levels of the tight junction protein, occludin [104]. Also autoantibodies against appetite-regulating peptides and neurotransmitters may disrupt the blood-brain barrier and the gut-barrier permeability in AN and BN [268]. Furthermore, gut-related antigens including gut microflora may influence production of specific autoantibodies (IgA class) against appetite-regulating hormones [47]. Indeed, starvation decreases the gut-barrier permeability in AN [102] and may decrease ghrelin autoantibodies (IgM, IgG, and IgA classes) production. However, realimentation-induced changes in the gut-barrier permeability and new antigenic stimulation during refeeding were accompanied by an increase of acylated ghrelin autoantibodies (IgM class) in AN [48].

behavior, appetite control, and immunoregulatory system in AN and BN. Indeed, Corcos et al. [43] hypothesized that dopamine, dopamine-beta-hydroxylase, and serotonin acting with autoantibodies could be the antigenic cerebral targets reacting with "anti-brain" antibodies in BN. All autoantibodies against dopamine, dopamine-beta-hydroxylase (i.e., the enzyme that synthesizes NE from dopamine), and serotonin were lower in BN than in the controls (Table 1). Moreover, the autoantibodies directed against feeding-stimulatory and feeding-inhibitory neuropeptides have been reported in patients with AN and BN. These autoantibodies correlated with psychopathological traits in individuals with eating disorders [44–46] and that neutralizing autoantibodies directed against appetite-regulating peptides were classified as important attributors to mechanisms controlling motivation in AN and BN (Figure 2). Fetissov et al. [47] studied healthy women for the presence of autoantibodies directed against 14 key appetite-regulating neuropeptides or peptide hormones including NPY, ghrelin, leptin, or PYY (Tables 1 and 2). Thus, these results confirmed that autoantibodies against hormones represent a general physiological phenomenon, suggesting its implication in physiological peptidergic transmission. The detection of the immunoglobulin (Ig) A class of such autoantibodies supports the antigenic stimulation by gut microflora in healthy subjects [47] (Figure 2). In fact, patients with AN display increased plasma levels of alpha-melanocyte-stimulating hormone (α-MSH) autoantibodies [45] contrasting with lower levels in acylated ghrelin autoantibodies [48] (Table 1). The presence of immune complexes sequesters autoantibodies against nonacylated ghrelin resulting in higher levels of free acylated ghrelin in AN patients, and eventually resulting in ghrelin resistance in AN [48]. Intriguingly, this is a potentially analogous situation in which autoantibodies against insulin may play a role in the shifts of bioavailable levels of insulin with possible effects on hypoglycemia. Involvement of insulin autoantibodies in insulin resistance has been extensively studied as a mechanism underlying insulin resistance after insulin administration [49] and autoantibodies against insulin have been studied as a marker of type 1 diabetes [50]. Using homeostasis model assessment of insulin resistance (HOMA-IR), we found significantly lower values of HOMA-IR in malnourished and underweight AN patients when compared with the controls [28, 29]. However, refeeding is associated with the onset of insulin resistance in AN patients [51].

The aim of this review was to describe the key role of orexigenic and anorexigenic hormones originating from the gut, central nervous system, and AT and to discuss how an impairment of energy balance and interaction between these factors and up- or downregulated neutralizing autoantibodies are involved in the pathogenesis, autoimmunity, regulation of food intake, energy expenditure, and growth hormone (GH) release in eating disorders. Understanding of the pathogenic mechanisms may contribute to more specific and effective therapy in AN and BN.

2. An Overview of Hunger-Satiety Signals and Autoantibodies against Appetite-Regulating Neuropeptides and Peptides of the AT-Gut-Brain Axis

2.1. Ghrelin. Ghrelin is a 28-amino acid peptide which increases food intake and acts as an endogenous stimulator of GH [52]. The ghrelin gene encodes a polypeptide preproghrelin containing 117 residues which undergoes stepwise processing to form ghrelin [53]. Ghrelin is predominantly produced by the stomach but is also expressed in many other tissues and is the first identified hormone containing acylated n-octanoic acid in its residues by ghrelin-O-acyl-transferase (GOAT) [54, 55]. This acylation is essential for binding to the growth hormone secretagogue (GHS) receptor type 1a (GHS-R 1a) and for the GH-releasing and appetite-stimulating activities. Genetic variation of GOAT is implicated in the etiology of AN [56]. Unexpectedly to the ghrelin secretion pattern, it was shown that GOAT mRNA levels decrease upon prolonged food deprivation and increase postprandially. Therefore, GOAT-ghrelin system may also act as a nutrient sensor by using readily absorbable medium-chain fatty acids to signal to the brain that high caloric food is available [57] and that dietary lipids can directly influence ghrelin acylation.

The effect of ghrelin on GH release is two to three times greater than that of GH-releasing hormone (GHRH) in humans [58]. Moreover, peripherally administered ghrelin signals *via* the vagus nerve to the brain where it triggers the release of GHRH and contributes to the activation of the food intake signalling cascade by NPY neurons in the arcuate nucleus of the hypothalamus [59]; when the vagus nerve is cut, the induction of GH release after ghrelin injection is dramatically decreased. It was documented that GH inhibits stomach ghrelin secretion. These findings indicate that the vagal circuit between the brain and the stomach has a crucial role in regulating plasma ghrelin levels [60]. Asakawa et al. [61] indicated that in contrast to acylated ghrelin, nonacylated ghrelin induces a negative energy balance by decreasing food intake and delaying gastric emptying. Nonacylated ghrelin present in plasma in far greater quantities than acylated ghrelin seems to be devoid of any endocrine action. However, it is able to exert some nonendocrine actions including cardiovascular and antiproliferative effects by binding different GHS-R subtypes [62]. It was described that acylated ghrelin [63] and nonacylated ghrelin pass the blood-brain barrier by means of transmembrane diffusion [64]. Nonacylated ghrelin induces an increase in neuronal activity in the arcuate nucleus, is involved in the regulation of the synthesis of anorexigenic mediators like urocortin and CART in the hypothalamus, and interacts with the corticotropin releasing factor receptor subtype 2 (CRF_2-R). Therefore, the neuromodulatory peptides CART and urocortin might thus play a key role in the anorexigenic effect of nonacylated ghrelin *via* CRF_2-R-dependent signalling [65].

The role of acylated ghrelin, nonacylated ghrelin, and other ghrelin gene-derived peptides in the postprandial regulation of satiety was not established. Recently, we found decreased levels of plasma total, acylated, and nonacylated ghrelin and obestatin after a high-carbohydrate breakfast in healthy women [66]. It is possible that obestatin may postprandially blunt the effect of ghrelin in healthy normal weight women.

Ghrelin increases food intake through effects on NPY [67]. Recently, studies in rodents suggested a possible mediation of ghrelin action on GH by NPY and that GH may be involved in maintaining feeding [68]. Plasma ghrelin levels are elevated during fasting and suppressed after meal. It was shown that the efferent vagus nerve contributes to the fasting-induced increase in ghrelin secretion and that higher ghrelin stimulates the afferent vagus nerve, promotes food intake, and contributes to ghrelin-induced GH secretion. These findings demonstrate that the vagal circuit between the brain and stomach has an important role in regulating plasma ghrelin levels [60]. Peripheral ghrelin signalling, which travels to the nucleus tractus solitarius (NTS) in part *via* the vagus nerve, increases NE in the arcuate nucleus of the hypothalamus, thereby stimulating feeding, at least partially through $alpha_1$- and $beta_2$-noradrenergic receptors [69]. Furthermore, ghrelin may have an integrative role in the behavioral and an adaptive response to starvation by increasing anxiety and alertness in animals and humans [70]. Ghrelin is also a potent secretagogue for GH and an intravenous (i.v.) ghrelin administration stimulates GH release in a dose-dependent fashion in humans [71], and there may be a positive association mediated by ghrelin, alternatively, a negative feedback action such that inhibition of plasma ghrelin levels occurs when plasma GH levels are high [72].

Fasting plasma ghrelin concentrations in humans are negatively correlated with body mass index (BMI) [73]. In obese individuals, dieting is associated with an increase in plasma ghrelin levels [74]. Both peripherally and centrally administered ghrelins produce a positive energy balance and lead to body weight gain [75]. In humans, acylated ghrelin induces a rapid rise in blood glucose and plasma insulin levels. However, coadministration of nonacylated ghrelin counteracts this effect. A separate i.v. administration of nonacylated ghrelin improves glucose metabolism and insulin sensitivity and inhibits lipolysis in humans [76]. Based on these data, Van Der Lely [76] suggests the existence of a specific receptor for nonacylated ghrelin other than CRF_2-R and GHS-R 1a.

Current analysis has the expression of ghrelin in a number of endocrine tissues such as AT [77]. Recently, Liu et al. [78] have explored the effects of ghrelin on the proliferation and differentiation of preadipocytes *in vitro* and confirm that ghrelin induces the differentiation of 3T3-L1 preadipocytes into mature adipocytes. Rodent and human studies indicate that ghrelin elicits an antilipolytic effect mediated by both acylated and nonacylated ghrelin and promotes adipogenesis [79]. However, predominant nonacylated ghrelin does not appear to activate GHS-R1a, and it remains unclear through which receptor nonacylated ghrelin mediates its action in AT, although it has been suggested that the antilipolytic effect of ghrelin could be mediated by an unidentified non-GHS-R 1a receptor [80]. Interestingly, the ratio of acylated and nonacylated ghrelin production might help to regulate the balance between adipogenesis and lipolysis in response to nutritional status [81]. Recently, Tebbe et al. [82] have shown that ghrelin

effects in the rat central nervous system appear to be mediated through receptor Y1, which also mediates the antilipolytic action of NPY_{1-36} and PYY_{1-36}. Thus, ghrelin may mediate its peripheral action in AT through Y1 receptor. Kos et al. [83] have demonstrated antilipolytic action of ghrelin in human AT and showed that acylated and nonacylated ghrelin may be ligands for Y1 mediating lipogenic effect in humans.

2.1.1. Ghrelin Levels and Autoantibodies against Ghrelin before and after Realimentation in AN and BN. Fasting plasma ghrelin levels have been reported to be increased in underweight patients with AN, especially in patients with binge-purge subtype of AN as compared to patients with restrictive type of AN, suggesting that binge-purging behavior has some influence on plasma ghrelin [84, 85] (Table 1). These findings were not confirmed by Otto et al. [86], who did not find difference in plasma ghrelin between restrictive and binge-purge subtypes of AN, and by Troisi et al. [87], who detected opposite results with higher plasma ghrelin levels in restrictive type of AN as compared to patients with binge-purge subtype of AN and BN individuals. Ghrelin is increased in the case of AN, and this increase in plasma ghrelin levels may occur either as an adaptive response to correct the abnormal energy status or as a result of relative resistance to ghrelin [88].

A greater fall of plasma ghrelin levels was seen in AN than normal controls following an euglycemic hyperinsulinemic clamp [89]. In women with AN, Karczewska-Kupczewska et al. [89] reported significant positive correlation between fasting ghrelin and insulin sensitivity and that the progressive decline in circulating insulin would favor ghrelin production in AN [84]. The enhanced plasma ghrelin levels of underweight AN patients tend to normalize after refeeding [90]. Furthermore, patients with AN do not show a decrease in plasma ghrelin following a standardized meal that is observed in healthy women [91], and anorectic patients would be refractory to the orexigenic action of ghrelin to regain a normal weight and replenish energy stores (Table 1).

Furthermore, our group assumes that the preproghrelin is cleaved differently in eating disorders such as AN than under physiological conditions. Thus, intact acylated ghrelin 1–28 is rapidly degraded to nonacylated forms or smaller fragments in AN patients. Alternatively, a loss of function mutations in GOAT might disturb the ratio of acylated ghrelin to nonacylated ghrelin [56]. These hypotheses are in keeping with the finding of Hotta et al. [92] who reported decreased levels of plasma acylated ghrelin in AN patients. However, Germain et al. [93] documented that the acylated ghrelin/total ghrelin ratio has been found to be increased in the restrictive type of AN, whereas it decreased in the binge-purge type of AN and BN.

GH levels are higher in patients with AN than in controls, and these higher levels are consequent to higher levels of ghrelin, a GH secretagogue. Thus, the hypersecretion of ghrelin might contribute to the hypersomatotropism of AN [94]. Indeed, a dysfunction of the ghrelin feedback systems might lead to the pathophysiology of AN and BN [60]. Furthermore, Støving et al. [95] suggest that GH hypersecretion in AN is due to decreased hypothalamic somatostatinergic tone restored by weight gain in these patients.

The physiological inhibitory role of free fatty acids (FFA) on GH secretion seems to be preserved in patients with AN. In fact, the infusion of FFA inhibited the elevated basal GH levels and abolished the exaggerated GH response to the GHRH, whereas the administration of antilipolytic drug Acipimox (Aci) led to the decrease in plasma FFA and markedly enhanced the GHRH-induced GH rise in patients with AN but not in healthy women [96]. Although patients with AN showed a hyperresponsiveness to GHRH administration [95], their GH response to ghrelin administration is surprisingly blunted [97]. This finding is consistent with desensitization of the GHS receptor induced by the chronic elevation of ghrelin levels in AN or impaired metabolic status in AN because ghrelin administration was not followed by increase in blood glucose levels in these patients [97]. Indeed, AN is associated with a nutritionally acquired resistance to GH with elevated GH levels, and low levels of the GH-binding protein indicate decreased expression of the GH receptor, which accounts for the state of GH resistance in the starved state [98]. This is consistent with results reported by Fazeli et al. [99] that administration of supraphysiological recombinant human GH in patients with AN does not overcome the state of GH resistance. Therefore, the administration of recombinant human GH was not associated with a significant change in plasma levels of blood glucose, insulin, or FFA in AN. Importantly, these findings suggest that patients with AN would have a relatively high resistance to the effects of GH [99]. In AN, a greater mobilization of FFA leads to an increase in the peroxisome proliferator-activated receptor-alpha (PPAR-α) which increases levels of fibroblast growth factor 21 (FGF 21) [100]. In fact, FGF 21 is a novel adipocytokine which may mediate GH resistance and reduces insulin growth factor 1 (IGF-1) levels in AN [101]. Patients with AN display low levels of autoantibodies against acylated ghrelin and higher levels of autoantibodies against nonacylated ghrelin present as immune complexes. Interestingly, the negative correlations were found between plasma autoantibodies and ghrelin peptides, and the decrease of bioavailable ghrelin autoantibodies may underlie an increase of plasma ghrelin levels and the resulting phenomenon of ghrelin resistance in patients with AN [48]. Indeed, starvation-induced changes decreased gut-barrier permeability [102] and may decrease ghrelin autoantibodies (IgM, IgG, and IgA classes) in AN. However, Terashi et al. [48] found that refeeding in AN patients was accompanied by an increase of acylated ghrelin autoantibodies (IgM class), which may indicate new antigenic stimulation leading to realimentation-induced changes in the gut-barrier permeability in AN patients (Table 1, Figure 2). In contrast to the decreased gut-barrier permeability during starvation in AN, increased levels of plasma FFA and the more ketone bodies produced increase the permeability of the blood-brain barrier during starvation and weight loss in patients with AN [103]. Moreover, starvation, stress, catecholamines, microbial antigens, poststreptococcal autoimmune process (PANDAS), and proinflammatory cytokines decrease blood-brain barrier integrity in parallel with decreased levels of the tight junction protein, occludin [104]

(Figure 2). Hence, it is possible that access of high affinity autoantibodies against appetite-regulating neuropeptides and peptides in the brain centers normally protected by the blood-brain barrier may trigger the development of AN and BN (Figure 2).

It is accepted that plasma levels of active acylated ghrelin represent less than 10% of circulating total ghrelin levels, which include acylated and inactive nonacylated ghrelin. We found increased plasma nonacylated ghrelin but not acylated ghrelin levels in AN patients (unpublished data). While high plasma total ghrelin in AN has been consistently observed [91, 105], elevated acylated ghrelin was found in few studies [106, 107].

It was reported that fasting plasma ghrelin was higher in the purging type of BN in comparison to the nonpurging type and in comparison to controls [108, 109]; this supports the idea that binge-purge cycles have an influence on fasting plasma ghrelin. However, subsequent studies did not detect any significant difference in plasma ghrelin levels between binge-purge BN patients and controls [12, 13, 87, 110] though Kojima et al. [111] found that BN patients exhibited elevated ghrelin levels despite higher BMI. In our recent studies, we reported increased response of GH and ghrelin to short-term exercise and antilipolytic drug Aci in BN patients and confirmed that GH exerted an inhibitory feedback effect on plasma ghrelin during exercise only in BN patients but in both BN patients and healthy women during exercise with Aci administration [12, 13]. Therefore, these data established ghrelin as a potential discriminator between women with eating disorders and healthy women [87]. Furthermore, the ghrelin responses to a standardized meal have been reported to be blunted in symptomatic binge-purge BN patients as compared to healthy controls [110–112]. However, in our recent study, we documented decreased ghrelin levels in BN patients after a high-carbohydrate breakfast [11] (Table 1).

In contrast with anorectic patients, the normal GH response to ghrelin administration was observed in BN patients, and ghrelin administration was followed by increase in blood glucose in BN [113]. These authors hypothesize that ghrelin hypersecretion may have a role in eating behavior but normal GH and blood glucose response to ghrelin administration may reflect less impaired nutritional status in BN patients [113].

2.2. Obestatin. Recently, it has been demonstrated that preproghrelin undergoes additional proteolytic cleavage, generating a 23-amino acid peptide, which has been named obestatin, and amidation of obestatin is likely essential for its biological activity as well as acylation of ghrelin. Responses to obestatin$_{1-23}$ were greater than those to obestatin$_{1-10}$ and obestatin$_{11-23}$ [114]. Unlike ghrelin, treatment with obestatin did not increase GH secretion [17]. Interestingly, obestatin antagonized GH secretion and food intake induced by ghrelin only when ghrelin and obestatin were coadministered [115]. Fasting obestatin levels were significantly lower in obese patients than in normal weight and anorectic women [116], and significant increase of both plasma obestatin and ghrelin levels was demonstrated with weight loss in obese patients [117]. In contrast to ghrelin, obestatin has anorexigenic effects, reduces gastric emptying, inhibits jejunal contractions, and suppresses body weight gain [17]. However, several recent studies performed in rats and mice under various experimental conditions did not reproduce these results [118, 119] and did not support the concept that obestatin is an opponent or counterpart of ghrelin. Indeed, Gourcerol et al. [118] proposed to rename obestatin to ghrelin-associated peptide. It was revealed that the action of obestatin on the secretion of insulin, glucagon, and somatostatin is the same as the action of acylated ghrelin [120].

Further studies showed that obestatin was involved in inhibiting thirst and vasopressin secretion [121], affecting cell proliferation [122], increasing the secretion of pancreatic juice enzymes [123], and inhibiting glucose-induced insulin secretion [124]. Although Zhang et al. [17] implied that amidation of obestatin is essential for obestatin activity, Van Dijck et al. [119] did not demonstrate any suppressive effects on eating and drinking after central administration in mice despite using amidated obestatin. Interestingly, Pan et al. [125] reported that obestatin is unable to cross the blood-brain barrier and is rapidly degraded in the circulation; this was confirmed by Vergote et al. [126]. Therefore, an alternative hypothesis is that obestatin exerts its effects on eating and drinking through direct interactions with the gastrointestinal system. Indeed, Zhang et al. [17] observed decreased contractile activity of jejunum muscle strips *in vitro* and suppression of gastric emptying *in vivo* after obestatin treatment. Thus, the inhibition of jejunal contraction could generate an afferent vagal signal to induce satiety in the brain. Obestatin is an interesting peptide but controversial gut hormone. It was concluded that obestatin exerts a dual effect on glucose-induced insulin secretion. At a low glucose concentration, obestatin potentiated the insulin response to glucose. At a high glucose concentration, obestatin inhibited the insulin release [127].

In our previous study, Sedláčková et al. [66] demonstrated that plasma obestatin levels decrease similarly to ghrelin after a high-carbohydrate breakfast in healthy women. A possible explanation of the simultaneous postprandial decrease of obestatin with ghrelin is that the function of obestatin may be to antagonize orexigenic ghrelin action after food intake. Interestingly, obestatin was positively correlated to total ghrelin, nonacylated ghrelin, and NPY. The positive relationship of obestatin with total ghrelin in the postprandial period indicates that these two cleavage products of one gene could act in a similar fashion to increase food intake. This idea is confirmed by the positive correlation between obestatin and orexigen NPY. However, this positive relationship of obestatin with the nonacylated ghrelin may correspond with the idea of a dual effect of obestatin.

Zhang et al. [17] reported that obestatin was the cognate ligand for the orphan G-protein-coupled receptor 39 (GPR39) based on the claim of its binding to human GPR39 with high affinity and specificity. However, recent reports indicate that obestatin is unlikely to be the endogenous ligand for GPR39 due to the lack of specific binding on GPR39 receptor-expressing cells and the absence of signal transduction pathway activation [128]. The native receptor for obestatin remains to be identified.

Recently, Zhang et al. [129] suggested that obestatin was a hormone capable of binding to GPR39 to regulate functions of gut and AT. Another observation revealed that the obestatin receptor GPR39 was upregulated in AT during fasting, whereas GPR39 levels were decreased in cultured mouse embryonic fibroblast cell lines (related to 3T3-L1) during adipogenesis [130]. A decreased obestatin receptor GPR39 expression in human AT was found in patients with obesity [131]. These findings suggest a possible role of the obestatin receptor GPR39 in adipogenesis. It was speculated that the obestatin receptor GPR39 could possibly play a similar role in the liver, adipose, endocrine pancreas, and gastrointestinal tract tissue regeneration and differentiation [132]. Furthermore, Granata et al. [133] documented that obestatin promotes beta cells and human islets survival by binding to glucagon-like peptide-1 (GLP-1), that is, the receptor via which incretins act. Very recently, Fujimiya et al. [134] supposed that obestatin may act on the obestatin receptor on vagal afferent nerve terminals, and CRF-R and urocortin-2 neurons in the hypothalamus may mediate the action of obestatin to inhibit the gastroduodenal motility via CRF_1-R and CRF_2-R in the brain.

2.2.1. Obestatin Levels in AN and BN. Recently, Sedlackova et al. [11] documented that fasting plasma obestatin levels were increased in both AN and BN patients compared to controls (Table 2). Monteleone et al. [135] found that underweight AN patients displayed increased plasma obestatin and ghrelin levels and an increased ghrelin/obestatin ratio compared with healthy women, which may suggest that the hunger signal of ghrelin is stronger than the satiety signal of obestatin. A limitation of this study is represented by the low number of AN-restrictive patients with respect to AN binge-purge individuals because in AN binge-purge subjects ghrelin levels have been reported to be higher than in AN-restrictive ones [109]. In addition, Zamrazilová et al. [116] failed to reveal any significant differences in plasma obestatin levels between restrictive type of AN and normal weight women, but the higher ghrelin to obestatin ratio in AN might reflect a long-term reduction in energy intake which could contribute to susceptibility of AN women to bulimic episodes. No significant changes in these parameters were detected in BN patients [135]. Moreover, Harada et al. [107] and Nakahara et al. [136] showed increased plasma obestatin and ghrelin levels in small groups of AN restrictive patients compared with age-matched healthy women. None of these studies, however, calculated the ghrelin/obestatin ratio.

Recently, Germain et al. [105] have reported increased plasma obestatin and ghrelin levels and decreased ghrelin/obestatin ratio in restrictive AN. The decreased ghrelin/obestatin ratio could facilitate the food intake restriction in these patients if obestatin inhibitory effects on food regulation were more validated.

However, Sedlackova et al. [11] reported that the administration of a high-carbohydrate breakfast induced a similar relative decrease in plasma ghrelin and obestatin in AN, BN patients and the controls suggesting a role of obestatin with rather orexigenic properties (Table 2). Moreover, we found that the ghrelin/obestatin ratio was lower in AN compared to BN and controls. We suggested that different plasma obestatin levels in AN and BN may have demonstrated their diverse function in eating behavior. Germain et al. [93] revealed that total and acylated ghrelin and obestatin circadian levels are increased in patients with AN restrictive type compared with the controls but decreased in patients with AN binge eating/purging type and those with BN. Recently, Uehara et al. [137] reported that an increase in energy intake leads to a decrease in plasma obestatin levels in patients with AN restrictive type (Table 2).

2.3. NPY. NPY is a 36-amino acid peptide that has potent orexigenic properties [138]. Experimental evidence indicates that NPY is the strongest orexigenic factor in the hypothalamic control of feeding behavior [139]. NPY's activity in cellular metabolism is mediated through binding to G-protein-coupled receptors, of which at least four subtypes exist in humans (Y1, 2, 4, and 5) and which are present in most peripheral tissues. The hypothalamic Y1, Y2, Y4, and Y5 receptors have all been hypothesized to mediate the orexigenic effects of NPY [140].

NPY coexists with catecholamines in the central and SNS and in the adrenal medulla [141]. In coculture with adipocytes, sympathetic neurons secreted NPY, suggesting cross-talk between the neural cells and adipocytes [142]. Furthermore, NPY-containing nerves are present in the gut of many species. Orexigenic peptide NPY participates in ghrelin and GH regulation pathways [143]. Coiro et al. [144] revealed that a somatostatinergic pathway is involved in the mechanism connecting physical exercise to NPY secretion in humans. Even though plasma NPY levels do not reflect NPY secretion in the central nervous system, there is no clear evidence that plasma NPY levels originate from peripheral sympathetic nerve secretion or the adrenal gland and/or AT during exercise in humans [145].

The i.v. administration of NPY has no effect on GH secretion in healthy humans [146]. However, stimulatory effects of NPY on GH secretion have been reported in prolactinoma and acromegalic patients [147, 148]. In addition, some of these authors also described inhibition of GH secretion by NPY [148].

The role of NPY can be considered as helping to coordinate protective antistarvation activity and preventing further depletion of existing energy stores. Antilipolytic effect of NPY may also regulate plasma FFA. As FFA regulate insulin sensitivity, an impairment in NPY's antilipolytic action could lead to changes in insulin resistance [149].

Intracerebroventricular injection of NPY appears to mediate upregulation of the key enzyme of lipogenesis: lipoprotein lipase expression and activity in AT [150]. It was recently found that NPY is synthesized in human AT and stimulates the proliferation and differentiation of new adipocytes [151, 152]. Thus, AT-derived NPY could cause a significant rise of plasma NPY levels and may mediate reduction of leptin secretion [151]. Although to date the role of most of these receptors in human AT is poorly understood, binding studies [25] have suggested that Y1 receptor may mediate the antilipolytic effect of NPY in AT. NPY_{1-36} is cleaved by dipeptidyl peptidase IV (DPP-IV) to generate the truncated

$NPY_{3–36}$, with which DPP-IV diverts affinity of NPY from Y1 to other receptors such as receptor Y5 whose function remains elusive [153]. DPP-IV inhibitors are therefore likely to enhance the antilipolytic action of $NPY_{1–36}$ as well as $PYY_{1–36}$ [153]. Furthermore, in order to better understand the interactions between sympathetic neurotransmitters and glucocorticoids in AT, Kuo et al. [154] treated sympathetic neural cells with dexamethasone upon which the expression of NPY and its Y2 receptor was more than doubled. Therefore, cortisol and the adrenergic activity seem to converge on the NPY-Y2 adipogenic system. Thus, adipose-derived NPY may have implications for central feedback of adiposity signals.

2.3.1. NPY Levels in AN and BN. NPY is one of the primary systems regulating the stress response, emotionality, and hormones relevant to AN and BN. It has been reported that NPY can attenuate specific behavior when the organism is stressed, and antistress effects of NPY are relevant to psychiatric conditions such as AN and BN [6, 155, 156] and that NPY has an anxiolytic and antidepressive behavior profile [157]. Interestingly, it is possible that NPY contributes to both binge eating and subsequent purging in BN because NPY itself has been demonstrated to induce an emetic response [158].

In reports with AN, basal plasma NPY levels in AN patients did not differ from the levels in the controls [159, 160]. Discordant data have been published concerning NPY levels in AN patients. Plasma levels of NPY were significantly lower in anorectic women than in the control group [161], and plasma NPY was decreased during treatment of anorectic girls. These changes do not correspond with increasing body weight suggesting dysregulation of appetite and body weight control mechanisms in AN [162]. The study by Sedlackova et al. [14] published during the preparation of this paper assumes that increased fasting NPY levels unchanged after a high-carbohydrate and high-protein breakfast indicate that NPY may be an important biomarker for disturbed eating behavior in AN and BN patients (Table 1).

Recent studies have suggested that NPY is not merely an "orexigen" but acts to stimulate behavior which precedes the food intake and actually inhibits intake per se [163, 164]. It was found that the treatment with NPY increased physical activity, decreased food intake and caused a loss of body weight in rats [165]. From this point of view, it is possible that AN patients are physically hyperactive and eat only a little food in spite of having depleted body fat and pathologically upregulated hypothalamic orexigenic peptides [165].

Plasma levels of NPY during symptomatic and remission phases of BN are unchanged compared with age- and weight-matched controls [38]. However, plasma concentrations of NPY in patients with BN were significantly elevated in comparison to controls [6, 11, 13, 14, 161] (Table 1). In our recent study, we revealed that antilipolytic drug Aci during short-term exercise further increases plasma NPY levels in patients with BN [6].

2.4. PYY. PYY is a 36-amino acid gut peptide belonging to the same family as NPY, and PYY has recently been discovered in the hypothalamus of the human brain. PYY is released from the endocrine L cells of the distal ileum and colon in response to feeding [166]. PYY in the circulation exists in two major forms: $PYY_{1–36}$ and $PYY_{3–36}$. $PYY_{3–36}$ binds with the greatest affinity at the presynaptic inhibitory Y_2 receptor and is a peripherally active anorectic signal. $PYY_{3–36}$ is the product of cleavage of the amino terminus residues by DPP-IV from $PYY_{1–36}$. PYY is able to cross the blood-brain barrier by transmembrane diffusion from the circulation [167]. Evidence suggests that the anorectic effect of peripheral $PYY_{3–36}$ may be mediated via the presynaptic inhibitory Y_2 receptor present on arcuate NPY neurons [168]. It was also shown that $PYY_{3–36}$ inhibited dopamine and NE release through the NPY Y2 receptors in the hypothalamus supporting a central anorectic effect of $PYY_{3–36}$ [169]. In contrast to peripheral $PYY_{3–36}$, centrally administered $PYY_{1–36}$ and $PYY_{3–36}$ increase food intake. PYY injection into the third, lateral, or fourth cerebral ventricles potently stimulates food intake in rodents [170]. Therefore, while circulating, $PYY_{3–36}$ may access the higher affinity arcuate nucleus Y_2 receptors, and the central feeding effects of $PYY_{1–36}$ and $PYY_{3–36}$ may be mediated by lower affinity Y_1 and Y_5 receptors [16]. Circulating PYY levels are low in the fasting state and rapidly increase postprandially when PYY is released into the circulation [171]. Either central or peripheral administration of PYY reduces food intake and body weight gain in humans [168]. The role of PYY in the regulation of energy balance in humans remains to be clarified; however, reduced caloric intake was demonstrated following infusion of PYY [168]. Moreover, PYY is known to cause nausea and emesis in some individuals [172, 173] which can contribute to subsequent self-induced vomiting in BN. A single infusion of $PYY_{3–36}$ is capable of reducing food intake in lean and obese humans and decreasing circulating ghrelin levels [174]. Thus, it appears that peripheral $PYY_{3–36}$ acts as a satiety signal regulating the termination of meal, partially by decreasing the production of the hunger-stimulating plasma ghrelin. In addition, in healthy subjects, there is a negative correlation between plasma levels of ghrelin, PYY, and BMI, respectively [111]. Also a negative association between PYY and leptin levels was described. It was suggested that PYY levels increase with weight loss and when plasma leptin is low [175].

It was shown that exercise can function as a physiological regulator of hormone release in appetite control and that levels of $PYY_{3–36}$ are positively correlated with exercise intensity [176, 177].

Furthermore, the antilipolytic effect of PYY and NPY has been shown in human adipocytes. Labelle et al. [178] have noted that the receptor Y1 mediates the antilipolytic effect of NPY and PYY in rat adipocytes. Current evidence supports that Y1 does not only bind to $NPY_{1–36}$ but can bind other ligands of the pancreatic polypeptide family with potentially higher binding affinities for $PYY_{1–36}$ [83].

2.4.1. PYY Levels in AN and BN. Studies of $PYY_{3–36}$ secretion in AN and BN are still scanty [179]. In patients with AN, basal plasma $PYY_{3–36}$ levels have been reported to be normal [180], increased [181–183] or reduced [184]. Moreover, in anorectic patients, plasma $PYY_{3–36}$ response to food intake has been detected to be time delayed [180] or increased [182] (Table 2).

After a partial body weight regain, the $PYY_{3–36}$ response to a test meal was not completely restored in AN patients [182]. Recently, Sedlackova et al. [14] documented that basal plasma PYY levels were similar in AN and BN groups and reached significantly higher values after high-protein breakfast compared with high-carbohydrate breakfast suggesting an important role of ingested macronutrient in plasma levels of PYY (Table 2).

In BN, basal plasma PYY levels increase to markedly high values during the phases of abstinence from binge eating and vomiting to return to control levels after recovery [185, 186]. However, we and the others found that fasting plasma PYY levels during symptomatic phase of BN were unchanged and comparable with age- and weight-matched healthy women [14, 187]. Recently, a blunted PYY response to food ingestion was reported in bulimic patients together with a decreased response of ghrelin [110, 111] (Table 2). What might be the role of PYY and ghrelin aberrations in BN? It has been demonstrated that BN patients exhibit impaired CCK secretion [188]. CCK, a satiety factor, is a stimulant of PYY secretion [189]. Hence, depressed PYY levels may result from reduced CCK secretion. Furthermore, both studies have confirmed a negative correlation between PYY increase and ghrelin decrease. The suppression of plasma ghrelin and the increase of plasma $PYY_{3–36}$ after meal may show the compensatory activation of peripheral signals promoting termination of food ingestion. Therefore, Kojima et al. [111] speculate that a gut-hypothalamic pathway involving peripheral hormonal signals, such as ghrelin and PYY, may be related to the pathophysiology of BN.

2.5. CCK. CCK is a member of the gut-brain family of peptide hormones [190]. CCK is secreted by the gastrointestinal system in response to food intake as well as by specialized neurons in the myenteric plexus and the brain [191]. A rise in circulating concentrations of CCK terminates feeding in rats [192]. I.v. CCK infusion decreases hunger and feeding in humans [193].

CCK is synthesized as a 115-amino acid prepro-CCK that is cleaved to generate CCK-58. CCK-58 is the largest circulating form of the hormone in humans. From the amino terminus of the peptide, it undergoes sequential proteolytic cleavage generating shorter peptides: CCK-39, CCK-33, CCK-22, CCK-12, and CCK-8. CCK-8 is the smallest fragment with complete biological activity [191]. CCK performs its numerous functions by binding to G-coupled CCK receptors located on the targets organs. Two different receptors have been identified: CCK-1 and CCK-2. CCK-1 receptors are abundant in the gut and in a few discrete brain regions (NTS, the area postrema and the hypothalamus), while CCK-2 receptors are expressed in the cerebral cortex, the hypothalamus, vagal nerve, spinal cord, and gastric mucosa [191]. It was confirmed that CCK released from the small intestine when nutrients enter the duodenum stimulates CCK-1 receptor on sensory fibres of vagal afferents that transfer signals to the NTS in the brainstem [194]; both ghrelin and CCK, after release from the gut, transmit starvation and satiety signals to the brain through the GHS-R 1a and CCK-1 receptors, respectively, located in the vagal afferents. Lesions of the vagus nerve or the NTS abolish the satiety effect of CCK [8]. The satiety hormone CCK activates adrenergic/noradrenergic NTS neurons. Hisadome et al. [195] suggest that epinephrine and NE act as anorectic signals at the level of the NTS. Central administration of CCK also inhibits feeding *via* the activation of CCK-2 receptors which are expressed in the ventromedial and paraventricular hypothalamic nuclei [196].

Chen et al. [197] showed that CCK-2 receptor knockout mice had increased body weight but smaller AT mass. Only CCK-2 receptors but not CCK-1 receptors are expressed in AT suggesting that CCK-2 receptors regulate fat metabolism and that CCK-2 receptor may play a role in adipose differentiation. However, Clerc et al. [198] reported that hyperphagia and increased fat deposition were observed in CCK-2 receptor deficient mice. Until now, little has been known about the role of CCK-2 receptors in AT.

2.5.1. CCK Levels in AN and BN. Some studies in AN patients have found elevations in basal plasma CCK levels [199, 200] as well as increased CCK release following a test meal in anorectic patients [201] (Table 2). Other studies have found that measures of CCK function in AN were similar to or lower than in control subjects [202–204].

However, BN patients exhibit impaired CCK secretion [188], and levels of CCK in BN are reduced during symptomatic phases of the disorder and also during a phase of initial recovery [205]. Patients with BN have diminished release of CCK following ingestion of a standardized test meal [187, 206] (Table 2). It has been suggested that the decreased CCK response to a meal may play a role in diminished postingestive satiety observed in BN and that may contribute to the perpetuation and frequent relapse of this disorder [207, 208] (Table 2).

2.6. Leptin. Leptin is a 167-amino acid protein known to suppress appetite and regulate energy expenditure, and it suppresses activity of NPY neurons and stimulates pro-opiomelanocortin (POMC)/CART neurons in the arcuate nucleus of the hypothalamus. Leptin is secreted exclusively by adipocytes [209], and leptin has also been found in the stomach [210] and the pituitary gland [211]. Nevertheless, AT remains its main source responsible for 95% of leptin production [212]. Leptin acts through the leptin receptor (OB-R), which is expressed in the hypothalamus and peripheral tissues such as the gut and AT. This ubiquitous distribution of OB-R underlies the pleiotropic roles of leptin [213]. Soluble OB-R represents the major leptin binding activity in human plasma [214]. During nutritional recovery, leptin increases and the OB-R decreases indicating that OB-R could be used as a valid biomarker for nutritional recovery [215].

Plasma leptin levels reflect both energy stores and acute energy balance. Circulating leptin levels are positively correlated with BMI and AT mass, and food restriction results in suppression of plasma leptin levels, which can be reversed by refeeding [16]. Peripheral leptin administration reduces food intake resulting in loss of fat mass [216]. It was suggested that leptin-induced increases in energy expenditure may reflect an activation of the SNS [217]. A report by Tang-Christensen

et al. [218] support that central leptin administration activates the SNS and that increases plasma NE levels in primates.

Adipocytes possess large numbers of GH receptors, and it was shown that GH directly regulates leptin gene production [219] and that hyperleptinemia may suppress ghrelin secretion [220]. Furthermore, leptin has been shown to play a stimulatory role in GH secretion in rats [221]; however, leptin is likely to exert an inhibitory action on GH secretion *via* a stimulatory effect on hypothalamic somatostatin activity in humans [95], and/or GH hyposecretion might be explained by a resistance to leptin action because hyperleptinemia might contribute to the GH hyposecretion of obese patients [222], and it is suggested that the malnutrition-dependent reduction of leptin levels may play a role in the hypersomatotropism of AN [94, 99, 223].

As shown by recent studies, leptin dose dependently inhibits ghrelin transcription *in vitro* [224] and decreases ghrelin release from isolated rat stomach [225]. These findings raised the possibility that hyperleptinemia may suppress ghrelin secretion in obese patients [226]. There is also an opposing relation that ghrelin hypersecretion is in conjunction with hypoleptinemia in AN [94]. The importance of leptin as adiposity signal to the brain is revealed by evidence that leptin inhibits the activity of orexigenic ghrelin-NPY network, whereas low plasma leptin levels upregulate the expression of NPY neurons which coexpress ghrelin receptors [220].

Furthermore, the production of leptin is influenced by several regulators, being stimulated by antilipolytic insulin and blood glucose but inhibited by sympathetic activity, lipolytic catecholamines, and FFA [227]. As reported by Frühbeck et al. [228], leptin appears to be involved in the regulation of AT metabolism by both inhibiting lipogenesis [229] and stimulating lipolysis [227].

2.6.1. Plasma, Soluble OB-R, and AT Leptin Levels in AN and BN. It was shown that leptin is the major hormone to trigger the adaptation of an organism to food restriction [230]. These findings indicate that the drop in leptin secretion associated with weight loss induced *via* a reduced energy intake is a major trigger underlying adaptation to starvation in AN [231]. In malnourished and underweight AN patients, plasma leptin levels are consistently found to be markedly lower than in normal weight controls [29, 30, 161, 179, 231, 232], and weight recovery in AN is associated with a trend toward, increases in plasma leptin levels [223, 233] (Table 2). In contrast, plasma soluble OB-R level was reported to be increased [232, 234]. This increase may reflect a protective mechanism that decreases free leptin bioavailability and thus further facilitates energy conservation in AN patients [232].

Interestingly, in our study, Dostalova et al. [232] reported significantly reduced plasma leptin levels but normal dialysate leptin concentrations in subcutaneous abdominal AT in AN patients. This finding could be explained by the increased number of smaller adipocytes in subcutaneous abdominal AT leading to a higher number of adipocytes per volume in AN patients when compared with the controls. Another explanation of this may be due to a reduced efficiency of both the SNS [179] and NPY inhibiting adipocyte leptin production in AN. On the other hand, because of reduced volume of subcutaneous abdominal AT, less leptin is secreted into plasma. It has been shown that SNS can exert tonic inhibitory action on leptin secretion and that adrenergic regulation may contribute to rapid decrease both of plasma leptin and insulin levels during exercise in AN and BN patients [6, 7, 30].

Recently, Fazeli et al. [99] have shown that administration of supraphysiological recombinant GH in patients with AN leads to significantly decrease in plasma leptin levels when compared with the placebo AN group.

In normal weight subjects with BN, plasma leptin levels have been reported to be either decreased [235, 236] or normal [234, 237] (Table 2). It has been reported that BN patients with a significantly higher number of daily binge/vomiting episodes hyposecrete leptin in spite of no changes in their BMI [6, 7, 238, 239]. Interestingly, we observed opposite changes in plasma leptin and ghrelin levels during the exercise with Aci administration and in the postexercise recovering phase in both BN patients and healthy women [6, 7, 12, 13, 240]. Plasma soluble OB-R was unaffected in BN patients when compared with the controls [234].

3. Conclusions

In AN, there is a characteristic excess of both feeding stimulatory and feeding inhibitory signalling, producing the "mixed" signals for satiety and desire to feed leading to failure of hypothalamic regulatory pathways [241]. AN may be consistent with a state of nutritionally acquired GH resistance [99] and with the mechanism of the state of ghrelin resistance in AN [48, 242]. The ghrelin autoantibodies could alter the feeding regulatory neurocircuitry and eating behavior by changing of the signalling of the hormone ranging from transport to neutralization resulting in the phenomenon of ghrelin resistance in AN patients [48] (Table 1, Figure 2). Very recently, Acres et al. [243] and Hornig and Lipkin [104] hypothesize that AN is an autoimmune disease and may also be associated with major histocompatibility complex (MHC) gene polymorphisms. Indeed, the development of type 1 diabetes in adolescence seems to place girls at risk for the subsequent development of AN and BN [244, 245]. Thus, autoimmune disorders are associated with increased secretion of leptin, whereas AN and BN are conditions of reduced leptin production. Hence, leptin could represent the "missing link" between autoimmunity and nutritional status. Also, BN is associated with an autoimmunity [43]. In BN patients, decreased levels of autoantibodies against serotonin (IgG class) may be involved with the lack of satiety. Also the decreased levels of autoantibodies against dopamine and dopamine-beta-hydroxylase (IgG, and IgM classes) could be implicated in the exaggerated hunger of bulimic patients [43] (Table 1, Figure 2).

Nevertheless, high ghrelin and low leptin concentrations suggest an orexigenic adaptive mechanism of appetite regulation in response to low food intake in AN [100, 175, 231] (Tables 1 and 2). Differences between central and peripheral secretion of hypothalamic neuropeptides, gut-related

peptides, adipocytokines, and the altered cosecretion of hormones with monoamines (NPY-NE; CCK-dopamine; CCK-serotonin; PYY-serotonin) were found in AN and BN [21–23, 37–39, 41, 42]. Indeed, obesity-prone rats have abnormalities in their dopamine system, a key component of hedonic regulation [246], and acutely ill BN subjects have a reduced striatal dopamine transporter availability as well as reduced hypothalamic serotonin transporter availability [247]. Gut hormones, such as PYY and ghrelin, in humans alter brain activation in these corticolimbic areas and higher cortical regions [248, 249]. Thus, patients with BN mainly had an excess of ghrelin with binge eating behavior and decreased anorexigenic signals by neurotransmodulator disturbances [38, 108, 110, 111, 175, 188]. These "mixed" signals could underlie bulimic binge eating behavior in which a relative increase in orexigenic and a decrease in anorexigenic signalling is characteristic (Tables 1 and 2). Ghrelin secretion in AN is higher in its cephalic phase and may eventually facilitate binge eating behavior in bulimic subjects [250].

Importantly, the mesolimbic reward system including the ventral striatum and ventral regions of the anterior cingulated cortex and of the orbitofrontal cortex has been proposed to play a pivotal role in the genesis of AN [23, 251] (Figure 1). Indeed, a functional magnetic resonance imaging (fMRI) study showed that the central striatal reward system in AN was hyperactivated upon processing of disease-specific stimuli [252]. Thus, changes in peptidergic neurophysiology occurring in the acute state of an eating disorder may play a pivotal role in the pathophysiology of the disorder by providing a possible link between motivated behavior, reward processes, cognitive functions, and energy balance [253, 254]. Although not consistently, hypoactivation of brain areas was documented in the mesolimbic reward system in women with active AN and weight-restored women with AN [255].

Therefore, these observations may contribute to the disruption of AT-gut-brain signalling system and neutralizing autoantibodies in eating disorders (Figures 1 and 2). In recent years, knowledge in the field of food behavior has widely increased, leading to the design of molecules targeted for pharmacological correction of eating disorders and weight control [256]. Also lower dosages of leptin could potentially ameliorate hyperactivity, depression, the metabolic profile, and reproductive function in acutely ill AN patients after weight regain [257]. At present, leptin, ghrelin, GOAT, NPY, tumour necrosis factor-alpha (TNF-α) downregulating agents such as dexamethasone and potentially Aci, or their synthetic analogs [6, 7, 13, 56, 256–258] as well as selective serotonin reuptake inhibitors (SSRI) and serotonin NE reuptake inhibitors (SNRI) may be useful agents for the modulation of food intake, especially serotonin-dopamine antagonists (e.g., Olanzapine) which have been found to be effective for treating AN [256, 259]. In the treatment of eating disorders, modified blood-brain barrier in AN and BN is a therapeutic target for delivery of any therapeutics to the central nervous system [103, 104, 260] (Figure 2). The circulating autoantibodies against appetite-regulating neuropeptides and neurotransmitters can be purified and used as monoclonal antibodies in AN and BN. Thus, pharmacologically active monoclonal antibodies would recognize not only the peptide but also the corresponding sequence in the native receptor [261]. Further research is required to investigate the gut-brain-AT orexigenic/anorexigenic agonists or antagonists [262] and the modifications of their pathways with receptors and neutralizing autoantibodies as well as clearance of peptide for potential treatment of eating disorders such as AN and BN in clinical practice [263–265]. Taken together, more data are needed to clarify the etiopathogenesis and pathophysiology of AN and BN. New hopes have arisen through by the recent progress in understanding the immunoneuroendocrine regulation of energy metabolism and feeding behavior because the current long-term pharmacological therapy of anorectic and bulimic patients is almost unsuccessful [266]. Eating disorders and depression are frequently associated. Indeed, an increase of plasma NPY levels may be due to a protective mechanism that prevents the exhaustion of energy reserves in BN and AN patients [6, 14]. Thus, Garcia et al. [267] supported NPY protective role in depression and that decreased plasma levels of NPY autoantibodies (IgG class) are relevant to altered mood while their increased affinities may participate in reduced appetite and body weight in depressive disorder (Table 1, Figure 2). We believe that results of this review may be helpful for better understanding participation of the AT-gut-brain axis peptides and neutralizing autoantibodies on pathophysiology of eating disorders such as AN and BN.

Abbreviations

AN: Anorexia nervosa
BN: Bulimia nervosa
AT: Adipose tissue
NPY: Neuropeptide Y
PYY: Peptide YY
PP: Pancreatic polypeptide
CCK: Cholecystokinin
NE: Norepinephrine
GH: Growth hormone
GHS: Growth hormone secretagogue
GHS-R: Growth hormone secretagogue receptor
GHRH: Growth-hormone releasing hormone
FGF 21: Fibroblast growth factor 21
FFA: Free fatty acids
OB-R: Leptin receptor
BMI: Body mass index
DPP-IV: Dipeptidyl peptidase IV
$CRF_{1,2}$-R: Corticotropin-releasing factor receptor subtype 1, 2
CART: Cocaine- and amphetamine-regulated transcript
GOAT: Ghrelin-O-acyl-transferase
3T3-L1: Preadipocytes
GPR39: G-protein-coupled receptor 39
GLP-1: Glucagon-like peptide-1
IGF-1: Insulin growth factor 1
NTS: Nucleus tractus solitarius
SNS: Sympathetic nervous system
Aci: Acipimox
Ig: Immunoglobulin

PPAR-α:	Peroxisome proliferator-activated receptor-alpha
i.v.:	Intravenous
SSRI:	Selective serotonin reuptake inhibitors
SNRI:	Serotonin norepinephrine reuptake inhibitors
MHC:	Major histocompatibility complex
TNF-α:	Tumour necrosis factor-alpha
α-MSH:	Alpha-melanocyte-stimulating hormone
HOMA-IR:	Homeostasis model assessment of insulin resistance
POMC:	Proopiomelanocortin
fMRI:	Functional magnetic resonance imaging
PANDAS:	Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infection
i.e.:	In other words.

Conflict of Interests

There is no conflict of interests.

Acknowledgments

This study was supported by Grant no. 303/03/0376 from the Grant Agency GA CR of the Czech Republic. Also, this study was supported by Grant no. NR/9158-3 provided by the IGA Grant Agency of the Ministry of Health, Czech Republic, and by the project of Ministry of Health (Czech Republic) for conceptual development of research organization 00023761 (Institute of Endocrinology, Prague, Czech Republic).

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 701967, 9 pages
http://dx.doi.org/10.1155/2013/701967

Review Article

Diet-Regulated Anxiety

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Received 4 June 2013; Accepted 11 July 2013

Academic Editor: Carlos Dieguez

A substantial proportion of noncommunicable disease originates in habitual overconsumption of calories, which can lead to weight gain and obesity and attendant comorbidities. At the other end of the spectrum, the consequences of undernutrition in early life and at different stages of adult life can also have major impact on wellbeing and quality of life. To help address some of these issues, greater understanding is required of interactions with food and contemporary diets throughout the life course and at a number of different levels: physiological, metabolic, psychological, and emotional. Here we review the current literature on the effects of dietary manipulation on anxiety-like behaviour. This evidence, assembled from study of preclinical models of diet challenge from gestation to adult life, supports a role for diet in the important connections between psychology, physiology, and behaviour. Analogous processes in the human population in our current obesogenic environment are likely to contribute to individual and societal challenges in this area.

1. Introduction

The quality and composition of the food we eat is under constant scrutiny, and there are numerous factors which affect our food selection and preferences. The disparate properties of the food we consume themselves act in a regulatory feedback system to impact on our future choices. One way in which food may influence subsequent choice is by affecting mood and consequently behaviour. Although it is recognised that mood can affect what we eat, here we consider how food affects our mood and, in particular, anxiety levels.

The fat and sugar content of contemporary diets and of processed foods, in particular, is a high profile issue for consumer groups, the media and health professionals. There is also increasing pressure from public health bodies and governments to reduce the amounts of fats and sugars, along with salt, in our diet. However, there may be unexpected consequences of such changes as our bodies may respond not only to the presence of these substances in our diets but also their absence, following a dietary manipulation. Withdrawal of dietary components has the potential to influence future food choices and also our emotional health. Generic, calorie restriction, and weight loss diets usually fail to produce the desired long-term outcomes, but increased awareness of the effects of the composition of our diet over both the short and the long term may increase our ability to adapt our diets successfully to improve both metabolic and emotional wellbeing.

Anxiety is linked to prevalence of disease [1]. People with mood disorders often have poor quality diets which are low in fruits and vegetables but high in fat and sugar [2]. Increasingly, modified diets are being used to treat behavioural and mood disorders such as attention deficit disorder, where diets low in sugar and high in fatty acids are recommended [3]. Children have less control over their food choice than adults as they are highly influenced by their parents [4]. However, children do have a reputation for knowing what they want to eat and being "picky" eaters, and high levels of anxiety have been shown to increase selective or fussy behaviour [5]. Dietary choice, whether it is quality/composition or quantity, is also affected in overweight adults who report increased calorie intake when they are under stress [6]. Stress can interact with eating behaviour in a number of ways regardless of an individual's normal eating habits [7].

Nutrition and anxiety can have both independent and interactive negative impacts on health. The life stage at which either of these factors becomes unbalanced impacts on the

Table 1: Common tests of anxiety.

Test	Indicator of anxiety	Additional uses/outcomes
Mazes (T maze, Y maze, elevated plus maze, O maze)	Decreased time in open (unwalled) sections	Activity, speed, and cognition
Light dark box	Decreased time in light box	Activity and speed
Open field	Less time in centre regions	Activity and speed
Sucrose preference	Decreased consumption of sucrose	Anhedonia
Locomotor activity	Decreased activity	Activity, speed, and rearing behaviours
Marble burying	Increased compulsive burying behaviour	Compulsion, impulsivity, and neophobia
Novel object	Decreased exploration of unfamiliar object	Activity, speed, memory, impulsivity, and neophobia

severity of the outcome. Consequently, insults which occur during critical sensitive stages in development, especially *in utero* or in early life may have long-lasting consequences. This emphasises the importance of maternal diet and diet during adult life, and their interaction with mental health. The aim of this review is to bring together existing knowledge of how food components affect anxiety at various stages of development, while highlighting some major gaps in our current understanding.

2. Measures of Anxiety

Tests for anxiety focus mainly on an animal's normal response to a novel environment or object(s) and measure changes in locomotion, exploration, and reactive behaviour (e.g., preference for enclosed and dark environments) as well as changes in acute behaviour such as grooming and rearing (see Table 1 for common tests). Tests for anxiety tend to be less challenging to the animal than those for depression, which often attempt to enforce behaviour such as swimming or an escape response. Detailed information on behavioural tests for anxiety and the differences between anxiety and depression tests can be found elsewhere [8]. A wide variety of behavioural tests are used in drug testing but, in studies investigating dietary effects, elevated plus maze (EPM) and open field (OF) are the tests most frequently deployed. By their very nature these tests are affected by subtle variations in setting, manipulation, and indeed the strain or sex of the animal. Detailed evaluations of the more common maze tests are available [9, 10], setting out the limitations of the tests and differences between them. It is difficult to compare results between tests since exposure time, habituation, and prior experience of the test are all likely to affect the outcomes.

3. Effects of Diet

Concern over obesity and metabolic disease, as well as their potential relationships with mental health disorders, has led to increasing emphasis on strategies to tackle poor eating habits. This has led researchers to investigate the emotional value of food, which may make an important contribution to diet choice [11, 12]. Dietary habits at various stages of life from gestation into adulthood have been connected not only to disruption of energy balance but also to attention, mood, behavior, and anxiety disorders.

3.1. Maternal Nutrition. Increased risk of developing numerous conditions including obesity, metabolic disease, mental health issues, and anxiety has been linked to poor early nutrition, particularly during critical stages of *in utero* development [13]. One example of a mental disorder which has been linked to obesity is attention deficit/hyperactivity disorder (ADHD), where mothers of children with the condition are twice as likely to be obese as mothers of children without the condition [14, 15], illustrating that maternal nutrition may affect the mental health and behaviour of offspring. However, there are likely to be confounding issues related to the mother's health in these studies, and this supports preclinical work currently being undertaken to identify the association between mental health and maternal nutrition as in animal models health can be closely monitored and food intake and diet closely regulated.

Various maternal diets in rodents have been shown to impact on circulating hormone levels which are likely to contribute to increased susceptibility to disease. Maternal high-fat diets increased circulating leptin and insulin levels [16], as well as desensitising the dopamine system [17], which affected the offspring's feeding behaviour. Another study using maternal high-fat (saturated or trans fat) feeding from 4 weeks prior to mating through until weaning, showed that while a diet high in saturated fat increased circulating leptin levels in both male and female offspring, anxiety, as assessed on the elevated plus maze (EPM), was increased only in male rats and apparently irrespective of the composition of maternal high-fat feeding [18]. This suggests that behavioural effects may have some sex specificity although this requires further investigation. Links between maternal diet and anxiety have also been associated with the mood regulating neurotransmitter, serotonin [19]. Peleg-Raibstein et al. showed increased anxiety in the EPM in a model of maternal high fat-feeding (dams fed 60% high-fat diet prior to mating through to weaning) and also suggest that this effect on anxiety is due to elevated serotonin in the ventral hippocampus as a result of the maternal high-fat feeding [20]. Again using 60% high-fat diet prior to mating through until weaning, Sasaki et al. showed that males decreased

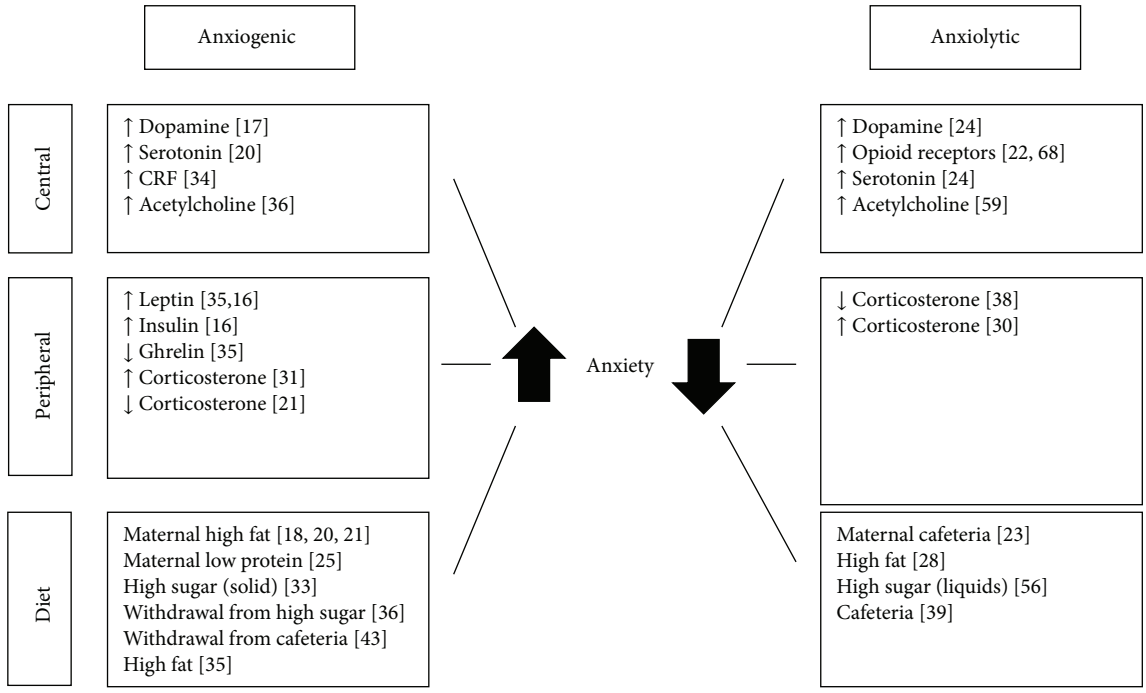

Figure 1: Factors implicated in mediating the effects of diet on anxiety. This illustrates some of the conflicting results from various palatable diet regimes and shows the complexity in deducing the pathways by which diets regulate anxiety levels. Numbers refer to associated referenced work.

time in centre of OF, females spent less time in open arms of EPM, but no differences were seen in the light dark box [21]. In terms of hormonal changes, this study also reported that offspring from dams on a high-fat diet had reduced corticosterone levels while the corticosterone receptors had increased abundance in the amygdala of females only [21].

Maternal diets composed of mixed palatable "junk" food or cafeteria feeding have been demonstrated to increase expression of opioid receptors and reduce dopamine transporters in offspring during early life. Although this effect appears to be reversible in later life, it suggests that the maternal diet is responsible for reduced sensitivity of opioid and dopamine signalling [22]. Also focusing on the effects of cafeteria diet, Wright et al. showed that dams fed cafeteria diet from weaning to mating produced male offspring that had reduced anxiety, increasing open arm exploration in the EPM and reducing latency to enter the centre of the open field [23]. Even providing this diet during the lactation phase only influenced dopamine and serotonin levels of the offspring [24]. There are numerous reasons why a cafeteria style diet might have different consequences to the high-fat diets described above. For instance, Figure 1 shows some of the central and peripheral factors which are affected by maternal diet and have been implicated as potential mediators of diet-regulated anxiety. The potentially high-sugar content of a mixed cafeteria style diet may also be responsible for counteracting the effects of the fat content or perhaps the more defining characteristic of a cafeteria diet; namely, the high variability and increased novelty and choice of food types may allow individuals to adapt their diet consciously or subconsciously to best suit their requirements to minimise anxiety.

Perturbations in supply of other macronutrients during early life may also impact on emotional behaviour, with low consumption of key dietary components such as protein affecting anxiety behaviour of offspring. For example, one study showed that protein restriction during pregnancy, but not lactation, increased anxiety as measured by EPM and decreased motivation for sucrose solution in operant system, but without effect on sucrose preference when given free access [25]. Likewise, another study of protein restriction from lactation onwards using foster dams found that anxiolytic drugs had little differential effect on anxiety of these animals in the EPM compared to rats on a standard diet, suggesting that any effects of malnutrition, in particular protein restriction, are likely to occur earlier in development [26].

3.2. High Fat. Alsiö et al. showed an inverse relationship between high-fat feeding of rats and anxiety in the EPM [27], and this was supported by A. Prasad and C. Prasad who found that diets high in fat but not those high in carbohydrates or protein were anxiolytic after just 1 week of dietary intervention [28]. In the light dark box paradigm, mice on a high-fat diet showed no response to an enforced stress whereas mice on a low-fat diet reduced the time spent in the light box by approximately 30% [29].

By contrast, Buchenauer et al. describe a reduced level of activity with changes in generic behaviour such as rearing (decreased) and grooming (increased) as indicators of

increased anxiety in rats following exposure to a 35% high-fat diet for 8 weeks [30]. Likewise, mice fed a 58% high-fat diet for 12 weeks in order to promote obesity have shown increased corticosterone prior to and following a stressor as well as anxiogenic responses in OF and EPM [31]. This raises the issue of the implications of dietary regime on baseline anxiety and on responses to subsequent exposure to stressful experiences.

As is frequently the case with dietary manipulations, the precise composition of the diet may be an important variable influencing the experimental outcomes. Accordingly, it has been suggested that saturated fatty acids are less anxiogenic than trans fats [32], so the source of fat in the diet may be as important as the quantity given. As indicated briefly above, both the quantity and type of fat in the diet vary across current reports on anxiety, along with the duration of feeding, all of which may impact on the anxiolytic or anxiogenic outcomes of high-fat diet feeding.

3.3. High Sugar. Solid chow diet enriched with sucrose appears to increase anxiety in rats as indicated by decreased time spent in the light chamber of a light-dark box although there was no effect on OF exploration [33].

Little work has been done on the direct anxiogenic properties of sugar in the diet although there is more information on the effects of subsequent withdrawal of sugar. Withdrawal of a liquid high-sugar chocolate-flavoured diet in rats increased anxiety on the EPM and also increased corticotropin-releasing factor (CRF) expression in the amygdala [34]. Interestingly, these rats were also hypophagic on standard chow. This same diet, even if provided for a short period of time (10 minutes) following a period of food deprivation, induced changes in hormonal levels and behaviour, with increased leptin, decreased ghrelin, and increased anxiety as measured in the EPM [35] suggesting that the anxiogenic effects of a palatable diet are fast acting and seen almost immediately after presentation of the food. The work of Avena and Hoebel and colleagues on withdrawal from sugar has been widely cited as a model of food addiction. With this model, rats habituated to intermittent feeding on sucrose were less willing to enter the open arms of the EPM after sucrose had been withheld suggesting an increased state of anxiety similar to that seen during withdrawal from addictive drugs [36]. It is interesting to note that this work has been done on withdrawal from sweetened liquids, and the form and schedule in which sugar is presented may affect quantities consumed and the extent of effects on anxiety.

Longer-term effects of a high-sugar diet were described by Souza et al. [33] who reported that after 4 months rats showed increased anxiety in the light/dark box but no effects in the OF setting. This again raises the question of the comparability of results from different behavioural paradigms and whether experimental examination of a greater range of tests could be revealing.

3.4. Mixed Palatable Diets. High-energy diets with a mixed composition of fat and sugar are known to result in memory deficits [37]. However, due to what may be opposing effects of fat and sugar on anxiety, as outlined above, it is difficult to predict their impact on behaviour and anxiety, and limited work has been done in this area.

Exposure to a "comfort" diet enriched with sugar and fat for just 6 days decreased corticosterone levels in rats following a foot shock stressor but did not affect basal levels. Anxiety behaviour was also decreased in an OF arena but no effects were observed either before or after stress in the EPM [38]. Likewise, stress responses to neonatal handling and early life isolation can be ameliorated by the anxiolytic effects of palatable diet high in fat and sugar [39], with increased locomotion and reduced anxiety in both the EPM and OF paradigms. This was confirmed in other work where stress was induced by isolation (maternal separation) and high-fat diets were reported to ameliorate anxiety in the EPM and light dark box in both dams [41] and pups [40–42]. It may be important that the diets were given in a cafeteria style and were also high in sugar.

Withdrawal of palatable (high fat, high sugar) diets has been shown to increase anxiety in obesity-prone animals in the OF paradigm as well as to increase motivation (operant lever pressing) for sucrose pellets compared to animals on the same feeding schedule but classed as being obesity resistant based on relative weight gain [43]. This would support the theory that preference for a diet or predisposition to consume a higher intake may interact with the primary diet effect and that effects on anxiety might be influenced by body phenotype rather than being solely diet dependent. Essentially, it appears that anxiety and diet and energy balance are likely to be interrelated. This will complicate attempts to distinguish between cause and effect, particularly with a mixed cafeteria style diet.

3.5. Irregular Eating. It is not only the components of the diet that affect anxiety behaviour. The frequency and regularity of feeding also affect circulating hormones and impact on behaviour due to effects on the circadian rhythms of the regulatory feeding and reward systems [44]. Models of irregular feeding can be considered to mimic the sporadic eating habits of dieters. Feeding a mixed high fat, high sugar diet to rats on an intermittent schedule either 2 hours a day or 3 days a week blocked the elevation of corticosterone associated with restraint stress seen in rats fed the palatable diet ad lib [45]. Rats given alternating access to a preferred high-sugar chocolate-flavoured diet had decreased activity and decreased time in the open arm of the EPM upon withdrawal from the preferred high sugar diet [46], and anxiety behaviour was greatest in those animals with the greatest propensity to binge. This may reinforce a cycle where mood or anxiety determines intake and the availability of certain foods feeds back on mood and behaviour.

4. Mechanisms

There are a number of possible mediators of the effect of diet on anxiety-like behaviour, including both peripheral and central mediators such as corticosteroids, insulin leptin, acetylcholine, serotonin, opioids and dopamine. The potential contribution of these factors is considered here in relation to the various palatable diets discussed previously.

The ability of maternal environment to influence the health status of offspring has been linked to various physiological changes associated with both over- and undernutrition of the mother [47]. There is evidence that hormones associated with energy status of the mother such as insulin and leptin as well as expression of neurotransmitters associated with reward such as dopamine can impact on anxiety and other behaviour disorders of offspring [48]. Also, the disregulation of serotonin, which also plays an important role in regulating neurogenesis and synaptogenesis and is affected by diet and stress [49], has been strongly linked to effects of maternal high-fat diet on anxiety in offspring [19, 50]. It has also been suggested that undernutrition of the mother alters the HPA axis of the offspring [51] and increases corticosterone [52], and these changes have all been associated with mediating the anxiety phenotype of the offspring. All of these factors may contribute to the overall impact that maternal diet can have on the anxiety levels of offspring and associated behaviour. Nutritional insult in early life may also influence the developing brain and behaviour and mental health in later life via epigenetic programming mechanisms [53].

In adults there are a number of common components of the stress, reward, and homeostatic pathways that link feeding behaviour, dietary choice, and anxious behaviour. While the hypothalamus is classically known for its role in the regulation of food intake and energy balance, corticosteroid receptors in this part of the brain are also known to be involved in the stress response, and this may provide a link between mood or anxiety levels and macronutrient preference or intake quantities [54, 55]. Circulating corticosterone is commonly measured as an indicator of stress and has been reported several times to be influenced by diet. Although it may be difficult to distinguish whether this is a result of rapid weight gain or a particular dietary component, the speed of the response would suggest the latter. A study by Ortolani et al. showed attenuation of corticosterone induction following stress after just 6-day exposure to a palatable diet compos of high fat and high sugar cafeteria style food although no effects of diet were seen on basal corticosterone levels [38]. Similarly, exposure to 30% sucrose solution had no effect on basal corticosterone but suppressed the anxiogenic effects of restraint stress [56]. By contrast, Buchenauer et al. describe elevated corticosterone levels in diet-induced obese rats following 8 weeks on a high fat-diet [30]. In mice there were no effects of HF diet on the HPA axis following a forced swim stress [29] although the diet did protect against stress-(social defeat) induced anxiety and depressive behaviour. Overall, this body of data appears to suggest that diet itself does not generically affect corticosterone or stress levels but may mediate the (protective) response to additional anxiogenic environments or stresses.

High-fat diets result in alterations to the circulating levels of insulin, and the resulting resistance to leptin has been associated with cognitive deficits in mice [57]. In a similar manner, mice provided with a 10% sucrose solution also became insulin resistant and had impaired cognitive function [58].

Morganstern et al. suggest a role for acetylcholine in the mediation of the effect of high-fat diet in reducing anxiety. These studies showed that high-fat diet increased open arm entries in the EPM and increased exploration of a novel object but, interestingly, also induced a reduction in acetylcholine esterase suggesting that high fat diet may increase neurotransmission via acetylcholine in the frontal cortex and hypothalamus [59]. In terms of sugar intake, acetylcholine has also been implicated in the generation of anxiety. Avena et al. described elevated acetylcholine levels in the nucleus accumbens as well as suppressed levels of dopamine, and suggested that these may be responsible for increased anxiety following withdrawal from sucrose [36]. Review of various types of anxiety disorders in humans and rodents has identified similarities in the activation of neuronal pathways in the medial prefrontal cortex [60, 61].

The central feeding pathways are known to contain orexigenic and anorexigenic neuropeptides which are responsive to changes in diet and energy status, but their role in behaviour, mood, and, indeed, anxiety regulation is less well understood. Recently, it has been shown that stress which results in anxious behaviour increases activation of POMC and AGRP in arcuate neurons [62], while ablation of POMC neurons in the hypothalamus creates a phenotype with increased anxiety-related behaviour on the EPM [63]. There is also some evidence that treatment with NPY can influence anxiety although contradictory findings make it difficult to determine the role it may play [64]. This would suggest that there is some potential for "traditional" feeding pathways to also mediate anxiety-related behaviour in response to diet.

Opioid pathways are strongly involved in preference for certain types of food, and, in a study where rats were divided into groups based on fat or sugar preference, responses differed depending on the opioid system being challenged [65]. Injection of opioid agonists increased fat preference, but when sweet foods were preferred over high-fat foods, neither the agonist, DAMGO, nor the antagonist, naltrexone, injected into the PVN affected the amount of preferred food consumed [65]. However, in a similar study looking at rats that had developed a food preference, the antagonist, naltrexone, injected into the amygdala, suppressed intake of the preferred food without affecting intake from other foods [66]. μ-opioid receptors have also been shown to play a crucial role in motivation for and liking of sugar (sucrose) and fat (corn oil), where an antagonist injected into the nucleus accumbens shell of rats produced fully reversible changes in feeding behaviour [67]. Likewise, opioids have a known role in anxiety; activation of delta opioid receptors suppresses anxious behaviour in the EPM [68]. For a review on the role of opioids in anxiety, see [69]. This confirms a dual role of opioids in mediating the intake of palatable foods and in regulating anxiety, making them likely facilitators of the effects of high-fat and high-sugar foods on anxiety-related behaviour.

Levels of dopamine receptor (D) expression within the nucleus accumbens are responsive to high-fat diet, with D1 levels decreasing and D2 levels increasing [31]. This would suggest that the ratio of these receptors may be important in mediating what is, in this case, an anxiogenic effect of

high-fat diet. High-sugar diets have also been found to affect dopamine receptor expression. In a model of intermittent exposure to sucrose Spangler et al. showed that there was also variation in the response of dopamine receptors in the nucleus accumbens to high-sugar consumption which decreased D2 and increased levels of D3 [70].

Figure 1 shows a summary of some of the potential mediators involved in diet-anxiety interactions. This highlights that there appear to be interactions between classical homeostatic feeding pathways, both peripheral and central, stress pathways, and central reward pathways in response to both diet and anxiety, which then give rise to behavioural outcomes. The exact relationships are however still unclear and further research is needed to identify the specific involvement of different brain regions and the hormones and neurotransmitters involved in these interactions.

5. Discussion

The effects of mood on eating habits and preferences have been investigated at a number of levels and over an extended period [71], but it is only recently that the focus of such investigation has been turned on its head through examination of the effects of diet and food components on mood, behavior, and future dietary preferences. Here, we have examined the effects of diet on anxiety-related behaviour and have highlighted that foods high in fat and/or sugar, or that are highly palatable, have the potential to impact on behaviour in animal models and, by extrapolation, in humans. Whether food modulates anxiety through anxiolytic or anxiogenic actions is likely to depend not only on its composition, but also on previous dietary experiences and maternal diet during and prior to pregnancy as well as contemporary stress-inducing experiences. This makes the role of food in regulating anxiety a highly complex topic, but also an area where increased understanding could lead to the development of strategies to use and adapt food and eating behaviour to beneficially address metabolic and mood disorders.

Currently, it appears that high fat is anxiolytic while diets high in sugar may have more anxiogenic characteristics. In terms of human lifestyle choices the situation is likely to be less clear cut, and mixed palatable diets are perhaps more relevant. Typically therefore, it is these diets which offer the greatest challenge of interpretation and manipulation, being highly variable in composition. Nevertheless, there is potential for these diets to be optimised and designed with beneficial effects for anxiety and general health.

Variation in mood affects food choice, shifting food preference towards foods high in fat and sugar [72], but, in the light of the evidence discussed above, this modification in preference may lead in turn to changes in anxiety and mood, thereby creating a feedback cycle. It is possible that in the wild, and across human evolution, consuming a high energy diet and being "cautious" could constitute beneficial behaviour conferring survival advantage and ensuring ingestion of sufficient energy supplies while reducing risk of predation. It can be speculated that ingestion of an anxiogenic food could lead to increased wariness and ingestion of familiar foods, whereas anxiolytic foods could increase exploration of the environment, and discovery of new palatable foods. Either scenario could have the potential to change behaviour and the mix of macronutrients and energy in the diet. In modern society with its wide range of food choice but where obesity and anxiety disorders are increasing, increased understanding of relationships with food at psychological and physiological levels including the associations between dietary choice and mood may be relevant to longer-term metabolic and mental health and be amenable to manipulation.

The research described above has all focused on common strains of laboratory rodents. It is important to remember that there are two reasons why an animal may be anxious. It may be predisposed to have an anxious character, for example, through genetic manipulation or selective breeding. This is known as trait anxiety. However, anxiety in response to stress or diet is known as state anxiety, and it is this form of anxiety that is most relevant here. However, it would be interesting to see whether responses to diet differ depending on the initial trait anxiety of the animal and, indeed, whether diet could be anxiolytic under these conditions. The choice of stock diets fed in such studies could then become important in terms of alleviating the side effects of drugs and improving animal welfare and even lifespan. In terms of diet-regulating anxiety, it is the basal levels of anxiety which are perhaps most interesting, but it will also be important, from the point of view of animal welfare, to establish the extent of the dietary effect on behaviour before challenging the animal to further stress or treatments.

Although research in this area is gathering momentum, a key issue in this and other related areas is the difficulty in comparing the outcomes of trials that involve differences in diet, species, strain, sex and life stage, and so forth, coupled to variation in duration, environment, and outcome measure. These issues have been aired in the context of diet-induced obesity in adult life [73, 74] and also apply to dietary impact on anxiety behaviour. Given the endless possibilities of diets that could be investigated under different feeding schedules, it would be pragmatic to focus on core dietary components in order to build up as complete a picture as possible. Even with the high profile already being afforded diets high in fat and sugar, there are still very large gaps in current knowledge. The effects of high-sugar diets during pregnancy, for example, have to date been studied to only a limited degree. Also, the quantities of fat or sugar needing to be consumed before there is a measurable effect, how long lasting is that effect, and how is it affected by trait anxiety and random stressful events remain to be determined.

The body of evidence reviewed here strongly supports the contention that diet has the potential to induce or ameliorate anxiety-related behaviour, but it remains to be definitively established how these events are regulated and whether there is potential to use these pathways to identify diets which may be beneficial in promoting healthy energy balance and mental health.

Acknowledgments

The authors were funded by the European Community's 7th Framework Programme under Grant agreement 245009 (NeuroFAST) and by the Scottish Government.

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Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 983964, 10 pages
http://dx.doi.org/10.1155/2013/983964

Review Article

Complementary Roles of Orexin and Melanin-Concentrating Hormone in Feeding Behavior

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Received 29 March 2013; Accepted 21 June 2013

Academic Editor: Carlos Dieguez

Transcribed within the lateral hypothalamus, the neuropeptides orexin/hypocretin (OX) and melanin-concentrating hormone (MCH) both promote palatable food intake and are stimulated by palatable food. While these two neuropeptides share this similar positive relationship with food, recent evidence suggests that this occurs through different albeit complementary effects on behavior, with OX promoting food seeking and motivation for palatable food and MCH functioning during ongoing food intake, reinforcing the consumption of calorically dense foods. Further differences are evident in their effects on physiological processes, which are largely opposite in nature. For example, activation of OX receptors, which is neuronally excitatory, promotes waking, increases energy expenditure, and enhances limbic dopamine levels and reward. In contrast, activation of MCH receptors, which is neuronally inhibitory, promotes paradoxical sleep, enhances energy conservation, reduces limbic dopamine, and increases depressive behavior. This review describes these different effects of the neuropeptides, developing the hypothesis that they stimulate the consumption of palatable food through excessive seeking in the case of OX and through excessive energy conservation in the case of MCH. It proposes that OX initiates food intake and subsequently stimulates MCH which then acts to prolong the consumption of palatable, energy-dense food.

1. Introduction

The hypothalamus has long been known to play an important role in feeding behavior. As far back as 1951, Anand and Brobeck [1] reported that bilateral destruction of the lateral hypothalamus (LH) in rats resulted in the complete absence of eating, leading them to term this area of the brain the "feeding center." Shortly thereafter, Delgado and Anand [2] reported that electrical stimulation of the LH in cats resulted in a 1,000 percent increase in total food intake. Interestingly, rats will work to receive electrical stimulation of the LH ("self-stimulation"), indicating that this nucleus also plays a function in reward, but excessive food intake leads them to decrease their rate of self-stimulation by half [3]. While a number of classical neurotransmitters have been implicated in LH-induced feeding, the discovery of neuropeptides in the brain [4] has led researchers to consider several of these local neuromodulatory neurochemicals as major players in feeding and reward.

Two neuropeptides transcribed in the LH are now understood to play significant roles in feeding and reward. The peptides, orexin A (OX-A) and orexin B (OX-B) (also called hypocretin 1 and hypocretin 2), are cleaved from the 130-amino acid precursor neuropeptide preproorexin (ppOX), which was independently isolated by two research groups in 1998 [5, 6]. Neurons containing orexin (OX) mRNA (about 6700 in the rat) [7] lie exclusively within the hypothalamus, spanning the dorsomedial hypothalamic nucleus through the perifornical area and into the lateral hypothalamic area [5, 6]. This peptide was immediately recognized for its ability to stimulate food consumption, leading one research group to name the peptide OX after the Greek word for appetite, *orexis* [6]. The peptide melanin-concentrating hormone (MCH), isolated from the salmon pituitary in 1983 as an antagonist of alpha-MSH-induced skin darkening [8], was recognized for its role in feeding in 1996 [9]. Neurons containing mRNA for the 165-amino acid precursor prepro-melanin-concentrating hormone (ppMCH, numbering about 12300 in the rat) [7]

are distinct from but adjacent to those containing OX, lying predominantly in the LH but also in the perifornical area and subzona incerta [10].

It is now well-established that OX and MCH can act as orexigenic neuropeptides, affecting both food intake and processes of reward that influence food intake. Despite their similar relationship with consumption, these peptides appear to act in complementary rather than redundant ways with food intake, and in fact have largely opposite roles in physiological processes and reward-related behavior. Here, we review the current knowledge about the relationship of these peptides with feeding, while providing a brief discussion of their other actions that may elucidate the mechanisms through which they promote food intake.

2. Receptor Function

2.1. Intracellular Effects of Receptor Binding. As with all neuropeptides, the receptors for OX and MCH are G protein-coupled receptors. There are two known receptors for OX, called the orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R), or the hypocretin 1 and 2 receptors. While OX1R binds to OX-A with an affinity that is two to three orders of magnitude greater than for OX-B, OX2R binds to OX-A and OX-B with nearly equal affinity [6]. Orexin receptor binding largely results in neuronal excitation, a rise in cytoplasmic calcium, with OX1R activating G_q subunits and OX2R activating G_q but also $G_{i/o}$ subunits [6, 11].

Depending on the species studied, there are either one or two receptors for MCH. Rats, mice, hamsters, guinea pigs, and rabbits have only one identified MCH receptor, MCHR1, but humans, rhesus monkeys, dogs, and ferrets also have a functional MCH receptor 2 [12]. As with OX2R, MCHR1 binding appears to activate both $G_{i/o}$ and G_q subunits [13], although the major effect of MCHR1 binding is a decrease in cyclic AMP levels [14, 15]. Thus, OX and MCH receptor binding has largely opposite effects on neuronal excitation.

2.2. Projections and Receptor Localization. Projections from OX- and MCH-containing neurons terminate in many of the same brain areas, which may explain why these neuropeptides affect a number of the same behaviors. These brain areas include the locus coeruleus, hippocampus, thalamus, nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala, cortex, and various nuclei of the hypothalamus [16, 17]. The receptors for OX and MCH are also located in these same brain areas [18, 19]. Interestingly, while OX1R and OX2R are often found in the same nuclei, they tend to predominate in different subregions of those nuclei. For example, in the hypothalamus, OX1R is most dense in the anterior hypothalamic nucleus while sparse in the LH and absent from the arcuate and paraventricular nuclei, and OX2R is sparse in the anterior hypothalamic nucleus while dense in the LH, arcuate, and paraventricular nuclei [18].

2.3. Interaction between Orexin and MCH. In support of the idea that OX and MCH work in a complementary or even opposite manner, these two peptides have been shown to interact directly with each other. Neurons containing OX-A or MCH are found to contact each other [20], and OX1R has been described on MCH neurons of the LH [21]. In slice preparation, the addition of OX-A or OX-B evokes long-lasting membrane depolarization and increases spike frequency of MCH cells [22], and the addition of MCH inhibits OX-A-induced spike frequency of OX neurons [23]. Therefore, while OX directly excites MCH neurons, MCH prevents excitation of OX neurons.

3. Physiological Effects

The OX and MCH systems largely play opposing roles in the regulation of the sleep-wake cycle and energy balance. Whereas OX promotes wakefulness and energy expenditure and is inhibited by a rise in glucose levels, MCH plays a role in sleep and energy conservation while being activated by glucose.

3.1. Role in Sleep-Wake Cycle. The firing of OX neurons is robustly tied to arousal during the sleep-wake cycle. These neurons discharge during wakefulness, cease firing with sleep onset, remain silent during slow-wave sleep, discharge periodically during paradoxical (or rapid eye movement, REM) sleep, and begin firing again prior to the transition from REM sleep to waking [24, 25]. They can fire during sleep when an arousing sound stimulus is presented [25, 26]. In support of a direct role for OX in promoting arousal, injection of OX-A or OX-B into the lateral ventricles increases waking and decreases slow-wave and REM sleep [27], while peripheral injection of the OX2R antagonist JNJ-10397049 but not OX1R antagonist SB-408124 decreases the latency for persistent sleep [28]. Transgenic mice lacking the ppOX gene display behaviors strongly resembling narcolepsy, exhibiting frequent periods of behavioral arrest during the dark (active) but not light phase [29]. In fact, the link between OX and narcolepsy was established soon after the neuropeptide's discovery, with an autosomal recessive mutation of OX2R identified in canine narcolepsy [30] and human narcoleptics found to have a reduction, as much as 95%, in the number of OX neurons [31]. Thus OX, acting through OX2R, functions to consolidate the waking state.

In contrast to OX, MCH has been linked with sleep, particularly with paradoxical sleep. Rather than discharging during wakefulness, MCH neurons fire maximally during REM sleep and occasionally during slow-wave sleep [32]. In support of a direct role for MCH in promoting sleep, injection of MCH into the lateral ventricles increases the quantity of paradoxical and slow-wave sleep [33], while both MCH and MCHR1 knockout mice show increased wakefulness [34, 35]. On the other hand, MCH does not appear to play a role in narcolepsy. Human narcoleptics show normal numbers of MCH neurons along with reduced numbers of OX neurons [31, 36], and there is no evidence to date linking mutations of MCHR1 to narcolepsy.

3.2. Role in Energy Balance. Orexin and MCH also largely play opposite roles in energy balance, in parallel with their

roles in physiological arousal. For example, intracerebroventricular (ICV) injection of OX potently increases oxygen consumption, and transgenic mice overexpressing ppOX are resistant to high-fat diet-induced obesity due to their increased energy expenditure and reduced fat consumption [37]. A specific role for OX2R in promoting energy expenditure is supported by evidence that this resistance to dietary obesity is found in OX overexpressors lacking OX1R but not in those lacking OX2R and that chronic ICV injection of [Ala11, D-Leu15] OX-B, which binds to OX2R, prevents the development of fat-induced obesity [37]. In contrast to OX, MCH promotes energy conservation. In addition to the small decrease in oxygen consumption that occurs with ICV injection of MCH [38, 39], mice overexpressing the ppMCH gene show increased body weight on a standard diet [40], while those lacking MCH show both decreased body weight and food intake [41, 42]. Interestingly, despite hyperphagia, mice lacking MCHR1 also exhibit decreased body weight on standard chow and are less susceptible to high-fat diet-induced obesity, likely as a consequence of their hyperactivity [43]. Also, genetically obese ob/ob and db/db mice are reported to have elevated MCH mRNA and peptide levels [44], and chronic ICV MCH increases caloric efficiency and body fat mass while an MCHR1 antagonist decreases them [45]. Together, these results support the idea that OX promotes energy expenditure, while MCH reduces it.

3.3. Regulation by Glucose. The activity of OX and MCH neurons is also regulated by energy status as indicated by levels of glucose. A physiological rise in glucose, which would occur after normal meal ingestion, is found to inhibit the electrical excitability of OX neurons in the mouse LH [46, 47]. This is in contrast to nearby MCH neurons, which are excited by elevated glucose levels [46, 48]. Whereas these two changes together might reflect a role for these peptides in energy balance, the evidence described below suggests that they may similarly be seen as promoting intake of a currently consumed food.

4. Role in Food Intake

Despite their discordant roles in behavioral state and energy balance, OX and MCH both act as orexigenic neuropeptides. While this effect can be seen with standard laboratory chow, it is even stronger with palatable food. A notable feature of palatable food is that it is generally overconsumed, such that homeostatic signals are overridden during the course of a meal, resulting in prolonged and excessive intake. Importantly, in addition to driving intake, both OX and MCH are themselves stimulated by the consumption of palatable food, further contributing to its overconsumption. While similar in this positive feedback circuit, the stimulation of food intake induced by OX and MCH appears to occur through different, complementary mechanisms. As described later, OX may increase the seeking and motivation to consume palatable food, whereas MCH appears to increase the reinforcing effects of caloric intake.

4.1. Effects of Peptides on Food Intake. A large body of evidence linking OX and MCH with food intake comes from studies of transgenic mice overexpressing or lacking the genes for these neuropeptides and also from studies of outbred rats using injections of the peptides or their antagonists.

4.1.1. Orexin. As described in the Introduction, the orexigenic effect of OX was noted at the same time that this peptide was first described [6], although the magnitude of its effect is much lower than that of the highly orexigenic neuropeptide Y (NPY) [49]. Under certain paradigms, OX can promote intake of standard laboratory chow in rats and mice. This has been shown for central injection of OX-A into the lateral ventricles [50], hypothalamic paraventricular nucleus [49, 51], LH or perifornical area [51, 52], as well as the NAc shell [53, 54]. While the feeding effects of ICV injection with OX-B are sometimes as potent as those of OX-A [50, 52], these effects of OX appear to occur primarily through OX1R, as a stimulatory effect on food intake with injections into specific brain sites has yet to be observed with OX-B [51, 52]. Notably, ppOX knockout mice show no difference in chow intake when compared to their wild-type littermates [55, 56], supporting the idea that this peptide may not be necessary for normal food intake.

A body of evidence indicates that the orexigenic effects of OX are far more robust with palatable food. In a variety of paradigms, peripheral administration of the OX1R antagonist SB-334867 is found to significantly suppress intake of palatable, high-fat food [57–59], and injection of OX-A into the third cerebral ventricle selectively increases intake of a high-fat diet more than a high-carbohydrate diet [60]. Whereas peripheral SB-334867 administration does not consistently decrease sucrose self-administration in rats [61–63], *ad libitum* fed ppOX knockout mice consume less of a sucrose solution than their wild-type littermates [64]. This link between OX and palatable food intake, similar to chow intake, appears to be mediated by OX1R, with the OX1R antagonist SB-649868 but not OX2R antagonist JNJ-10397049 found to decrease binge eating of a high-fat, high-sucrose food in female rats [65].

The ability of OX to increase food intake may occur in large part through its stimulation of arousal as well as an increase in motivation, particularly when palatable food is involved. While similar to wild-type littermates in their chow intake, ppOX knockout mice exhibit deficits in their ability to learn about the availability of food. Under mild food restriction, they demonstrate delayed acquisition of operant responding for chow [56] and significantly diminished food-anticipatory activity prior to scheduled feeding [55, 66]. Interestingly, conditional OX gene knockdown via RNAi in normal mice causes decreased responding for chow under both variable and progressive ratio schedules [56], suggesting that OX normally promotes reinforcement-related aspects of food intake. The particular reinforcement-related aspect may be motivation, as OX1R binding largely appears to affect palatable food intake by increasing the motivation for food. Peripheral administration of the OX1R antagonist SB-334867 decreases progressive and fixed ratio responding for a

high-fat diet and for sucrose [57, 61, 62], while third ventricle injection of OX-A increases progressive ratio responding for sucrose [57]. Similarly, SB-334867 significantly decreases cue-induced reinstatement of sucrose seeking [61]. Together, these results suggest that OX, acting at OX1R, promotes food intake by increasing the motivation for food reward.

4.1.2. MCH. The orexigenic effect of MCH on standard chow is roughly of the same magnitude as OX [49]. Intake of chow is increased in rats and mice after injection of MCH into the lateral or third ventricles [9, 49, 67], hypothalamic paraventricular nucleus [68], and NAc shell [69], while it is decreased after injection of an MCHR1 antagonist in the NAc shell [70] or periphery [71]. Although adult ppMCH and MCHR1 knockouts compared to wild-type mice actually demonstrate hyperphagia [41, 43, 72], there is evidence that ppMCH knockouts at a young age consume significantly less chow [41, 42], indicating that MCH has some role in controlling intake of standard food.

Similar to OX, the orexigenic effect of MCH is more robust with palatable food, particularly with calorically dense food. ICV injection of MCH promotes the intake of a high- or medium-fat diet, more than a chow diet [45, 73, 74], and ICV or peripheral injection of an MCH antagonist decreases intake of and operant responding for these diets [45, 75, 76]. Further, transgenic ppMCH overexpressors compared to wild-type mice exhibit increased consumption of a high-fat diet [40]. A similar relationship for MCH is seen with sucrose, with ICV MCH stimulating intake of sucrose solutions [77–79] and systemic administration of the MCHR1 antagonist GW803430 decreasing sucrose self-administration [80]. Notably, the relationship of MCH with palatable food does not extend to sweet, noncaloric saccharin [79, 80], indicating that MCH may be related more to energy conservation than to the intake of palatable food *per se*.

The ability of MCH to stimulate food intake may be due more to its reinforcement of ongoing intake rather than to an effect on the motivation to eat. This is supported by evidence showing that mice lacking the ppMCH gene show decreased responding for a high-fat diet under both fixed and progressive ratio schedules [81] and that Wistar rats given systemic injection of the MCHR1 antagonist GW803430 show reduced fixed and progressive ratio responding for a sucrose solution [80]. Further, although the same injection was found to suppress cue-induced reinstatement of lever pressing for sucrose [80], MCH blockade does not affect reinstatement for fat. Neither peripheral injection of the MCHR1 antagonist SNAP 94847 in rats nor chronic loss of ppMCH in mice significantly affects either cue- or pellet-induced reinstatement of fat seeking [58, 81]. Together, this evidence indicates that MCH promotes food intake primarily by increasing energy conservation, motivating animals to continue consuming energy-dense foods.

4.2. Effects of Food Intake on Peptides. Another set of studies that tie OX and MCH to food intake examines the effects of various feeding conditions on their levels of mRNA or peptide.

4.2.1. Orexin. Neurons containing OX are activated in anticipation of feeding but also following consumption of food, provided that it is palatable food. Food deprivation upregulates gene expression and protein levels of OX (OX-A and OX-B) and both of its receptors in the hypothalamus [82] as early as twenty-four hours after onset. After a forty-eight hour fast, similar changes have been observed [6, 83], and in female rats, the activity of OX neurons is also upregulated, as indicated by double-labeling of OX with phosphorylated CREB [84]. Given the relationship of OX with glucose (see Section 3.3), these changes after food deprivation may reflect lowered glucose levels; however, they may also reflect changes in arousal, as a single day of food deprivation increases wheel running activity [85] and decreases the total number of sleep episodes [86], effects that occur under conditions of heightened OX activity and are taken to indicate foraging behavior.

In line with the effects of food deprivation, OX neuronal activity and gene expression are also upregulated when animals are expecting to receive valued food. In rats with restricted access, OX mRNA and double-labeling of OX with c-Fos are elevated prior to access to a daily meal of chow, corn oil, or chocolate [57, 87], while OX levels begin to return to baseline within 30 minutes after the start of meal consumption [87]. In sated rats, OX and c-Fos double-labeling is also increased by a tone that signals the availability of palatable food in the form of high-sucrose pellets [88]. Siegel and colleagues [89] demonstrated that the expression of Fos in OX neurons increases in animals working for chow during the light phase but not when working to avoid shock or when receiving unearned rewards. These results suggest that OX neurons are activated in conditions when animals expect to receive specific, often preferred foods. In further support of this idea, c-Fos expression in OX neurons is also increased during extinction of sucrose seeking [61], when animals are motivated to obtain food rewards.

After consuming palatable food, OX levels are similarly elevated. With high-fat compared to low-fat, high-carbohydrate food, OX gene expression, and OX-A peptide levels are elevated after a single meal or up to three weeks on the diet [90–92], with longer periods of exposure leading to compensatory decreases in OX [93]. Interestingly, this fat-induced increase in OX may occur more from saturated than unsaturated fat, as consumption of a lard-based diet leads to higher OX mRNA levels than does that of a fish oil-based diet [94, 95]. Similar to the results with fat, OX gene expression is also elevated following consumption of a high-sugar diet [96]. Together, these findings indicate that OX is activated both when animals are seeking food and also after they have consumed palatable food, which may in part explain why these foods are consumed in excess.

4.2.2. MCH. Unlike neurons containing OX, those containing MCH are not consistently activated in anticipation of feeding, although they are activated following consumption of a palatable, caloric food. Gene expression of MCH is upregulated after twenty-four hours of food deprivation [9, 97], although longer periods of fasting either increase peptide levels or leave them unchanged [97–99]. The failure

to observe an increase may be due to the sensitivity of MCH neurons to the sleep-wake cycle. This is indicated by evidence showing MCH peptide levels after forty-eight hours of food deprivation to be increased in rats sacrificed during their resting (light) phase, but not their active (dark) stage [100] when MCH neurons are normally quiescent. Alternatively, MCH may play a less prominent role than OX in food seeking induced by deprivation, particularly as MCH neurons are excited by elevated glucose levels (see Section 3.3). Thus, these changes in MCH in response to food deprivation, unlike with OX, may be related more to changes in caloric efficiency than in circulating glucose.

In contrast to OX, there is little evidence linking MCH with the expectation of receiving food, with double-labeling of c-Fos and MCH in sated rats unchanged by a tone signaling the availability of high-sucrose pellets [88].

Levels of MCH after consuming calorically rich food are clearly increased, supporting its role in palatable food consumption. With maintenance on a high-fat compared to low-fat diet, MCH gene expression and hypothalamic peptide levels as well as hypothalamic MCHR1 mRNA levels are elevated [101, 102]. Although the type of fat in the diet may not make a difference in this effect [95], the caloric content appears to be essential, with drinking of noncaloric saccharin having no effect on MCH gene expression [103]. Thus, consistent with its proposed role described previously in promoting motivation for intake of caloric food, these results indicate that MCH is activated after animals have consumed caloric food, further promoting overconsumption.

5. Interactions with Other Neurochemicals

Whereas OX and MCH each play a significant role in the consumption of palatable food, it is clear that these neuropeptides do not work in isolation. Two classes of neurochemicals with which they directly interact are first-order feeding neuropeptides of the arcuate nucleus and dopamine in the limbic system.

5.1. Arcuate Peptides. The orexigenic actions of OX and MCH may be due, in part, to their similar downstream activation of neuropeptides in the hypothalamic arcuate nucleus, NPY and agouti-related protein (AgRP). In the arcuate, OX-positive axon terminals directly contact neurons containing NPY [104], and OX1R protein is located on both NPY and AgRP-containing neurons [21]. Orexin also directly activates NPY neurons, as the addition of OX-A or OX-B to the superfusate of isolated arcuate NPY neurons increases their intracellular calcium content [104]. This translates into effects of OX on feeding, as the orexigenic effect of ICV OX-A or OX-B is greatly reduced by ICV pretreatment with an NPY receptor antagonist [105, 106]. Similarly, chronic ICV injection with MCH, which stimulates feeding, also upregulates NPY gene expression [107], and the orexigenic effect of ICV MCH is significantly diminished by ICV injection of an NPY receptor antagonist [108]. In part then, the similar ability of OX and MCH to promote food intake may be due to their similar downstream effects on other orexigenic neuropeptides.

5.2. Limbic Dopamine. The dissimilar motivation and arousal-related actions of OX and MCH may be due more to their opposing downstream effects on the neurotransmitter dopamine (DA), in limbic nuclei such as the NAc and prefrontal cortex (PFC). A rise in OX levels results in DA release into the NAc shell and PFC, which can occur from OX acting directly at DA terminals or at their source in the VTA. Application of OX-A to PFC slices increases phasically evoked DA release, an effect inhibited by the OX1R antagonist SB334867 [109], and application of OX-A or OX-B to VTA slices increases the firing frequency of DA neurons [110]. Similarly, ICV injection of OX-A stimulates c-Fos in VTA DA neurons, primarily in those projecting to the NAc shell and PFC rather than the NAc core [111]. In line with this, ICV OX-A is also found to elevate levels of DA in the PFC but not the NAc core [112]. These findings with OX contrast with those observed with MCH, which through transgenic studies appears to inhibit accumbal DA. Mice lacking ppMCH exhibit increased electrically evoked DA release in the NAc shell and increased DA transporter levels in both the NAc shell and core [81, 113]. As the application of MCH to VTA slices fails to affect firing of DA neurons [110], these DA changes in knockout mice may be due to presynaptic actions in the NAc. These opposite effects of OX and MCH on limbic DA may help to explain their opposite effects on arousal and reward (see Section 6).

It is interesting to consider the similar orexigenic actions of OX and MCH in light of their opposite effects on DA. This neurotransmitter plays an important role in promoting food-seeking behavior [114] and appetitive motivational processes in general [115], but animals will also work to enhance DA levels when they are low [116]. In fact, animals prone to overeating a high-fat diet exhibit markedly reduced basal levels of DA in the NAc [117]. Seemingly then, palatable food intake can be increased from an OX-induced elevation of DA, which increases arousal and seeking of palatable food [118, 119], but it can also be increased by an MCH-induced reduction in DA, which produces anhedonia (see Section 6.2) and the need to restore DA levels through the consumption of palatable food [117]. This suggests the possibility that OX may contribute to the rise in accumbal DA that normally occurs prior to meal consumption, while MCH may contribute to the fall observed during the feeding bout [120].

6. Role in Reward

In line with their opposite effects on limbic DA, OX and MCH largely have opposite effects on processes of reward. Whereas OX increases the reinforcing properties of ingested substances, MCH instead appears to promote depression and anxiety.

6.1. Orexin. The peptide OX plays a role in reward and reinforcement, acting through OX1R or OX2R. In tests of conditioned place preference (CPP) following pairing with drug administration, VTA injection of OX-A during conditioning induces morphine CPP in a dose-dependent manner [121], and peripheral administration of the OX1R antagonist

SB-334867 or OX2R antagonist TCS-OX2-29 suppresses its acquisition and expression [122, 123]. While morphine CPP may be perpetuated by OX binding at OX1R or OX2R, ethanol CPP appears to be mediated more by OX2R. Acquisition, expression, and reinstatement of ethanol CPP are attenuated by peripheral treatment with the OX2R antagonist JNJ-10397049 but not by the OX1R antagonists SB-408124 [124] or SB-334867 [125]. The reinforcing effects of naturally rewarding stimuli may also involve OX. In male rats, conditioned cues associated with sexual behavior in a CPP paradigm induce c-Fos in OX neurons, and lesioning of OX neurons prevents the formation of CPP for a chamber paired with sexual behavior [126]. Thus, OX may be important in reward processing of both drugs of abuse and also natural rewards, such as sexual activity or palatable food intake.

6.2. MCH. In contrast to OX, MCH may not promote reward processing but instead is linked with anxiety and depression. Mice with deletions of MCHR1 show no difference from their wild-type counterparts in cocaine- or amphetamine-induced CPP [127] and in fact show hypersensitivity to the locomotor activating effects of the DA psychostimulant d-amphetamine [128], suggesting that these mice have enhanced drug-induced reward processing. Instead, MCH is strongly linked with anhedonia in the forced swim test (FST). The amount of time spent immobile in the FST correlates positively with ppMCH mRNA [129], and MCHR1 antagonists produce an antidepressant effect in the FST when administered peripherally [130] or directly in the NAc shell [70, 131]. Conversely, NAc shell injection of MCH produces the opposite effect [70]. The MCHR1 antagonist SNAP-7941 also acts as an anxiolytic in tests of rat social interaction and guinea pig maternal-separation vocalization [130]. Thus, in contrast to OX, MCH may in some cases act as an antireward neuropeptide.

7. Conclusions

In summary, OX and MCH, expressed in the LH and acting through their receptors in many of the same nuclei of the brain, are similar both in promoting the consumption of palatable or caloric food and in being stimulated by the intake of this food, responding in a positive feedback cycle to promote excess consumption. Importantly, they appear to have complementary roles in controlling this feeding behavior, likely through their largely opposite roles in reward systems and motivation, as well as a number of physiological processes, including the sleep-wake cycle, energy expenditure, and glucose metabolism. Perhaps for these reasons, OX and MCH affect different aspects of food intake. The evidence suggests that OX is activated in situations of food seeking and promotes the motivation for food reward, possibly activating the feeding process, whereas MCH plays a larger role during ongoing food intake and reinforces consumption, acting to prolong the intake of energy-dense foods. Thus, for a single meal, OX may be involved in initiating palatable food intake and activating MCH neurons, and after meal initiation and the consequent rise in glucose, MCH functions to prolong the consumption of calories for the sake of energy conservation. These physiological and behavioral effects of OX and MCH can be further understood in light of their downstream effects on other neurochemicals. While these neuropeptides both upregulate NPY which may contribute to their similar orexigenic effects, they have opposite effects on limbic DA, which may contribute to their complementary effects on reward and motivation that themselves can enhance palatable food intake. These two peptides in the LH provide an interesting example of the complex and sometimes opposing physiological, behavioral, and neurochemical processes that are involved in promoting the ingestion of palatable food.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This research was supported by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health under Award nos. K99AA021782 (JRB) and R01AA12882 (SFL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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