
HOT TOPICS IN ENDOCRINE AND ENDOCRINE-RELATED DISEASES

Edited by **Monica Fedele**

Hot Topics in Endocrine and Endocrine-Related Diseases

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Contributors

Carmen Sanz, Veronica Hurtado, Isabel Roncero, Enrique Blazquez, Elvira Alvarez, Imre Zoltan Kun, Zsuzsanna Szanto, Jozsef Balazs, Anisie Nasalean, Camelia Gliga, Cristina Carnovale, Paola Ingaramo, Daniel Francés, M. Teresa Ronco, Ebe D'Adamo, Benjamin U. Nwosu, Vania Maria Correa Da Costa, Antongiulio Faggiano, Manuel Estrada, Carla Basualto-Alarcón, Enrique Jaimovich, Rodrigo Maass, Marie-Lise Jaffrain-Rea, Emrah Yerlikaya, Fulya Akin

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Preface

The endocrine system plays a vital role in whether or not you develop a wide range of diseases, not only including strictly endocrine-diseases.

This book includes selected contemporary topics representing exciting recent developments in the field of endocrine and endocrine-related diseases. It is the result of a collaboration among eminent scientists and clinicians coming from eight different countries, including Argentina, Brazil, Chile, Italy, Romania, Spain, Turkey and United States.

Basic and advanced recent trends and research studies in a number of endocrine and endocrine-related disorders are covered by the different chapters of the book. We will read about hereditary neuroendocrine tumors, with emphasis on their still poorly defined natural history and clinical course; pituitary tumors, with a comprehensive overview of the recent advances in their pathogenesis; thyroid dysfunctions, including those related to aging and iodine deficiency; diabetes, with its hepatic complications, and double diabetes, a new definition that summarizes the coexistence of features of both type 1 and type 2 diabetes in the same individual, with a comprehensive discussion of the various aspects of this disorder and a focus on the search for a treatment paradigm in children and adolescents. Moreover, we will read about the testosterone as a new player in metabolic disorders and as a potential drug to counteract skeletal muscle diseases; the glucagon-like peptide type 1 and its multiple actions for which it can be considered an anti-diabetogenic agent and may have important implications in pathophysiological states such as obesity; the insulin-like-growth-factor role in different human pathologies, including cancer; and the pediatric nonalcoholic fatty liver disease.

I hope that, at the end, the reader will have a critic and advanced overview of selected hot pathologies related to the endocrine field.

Dr. Monica Fedele
Institute of Experimental Endocrinology and Oncology,
National Research Council,
Naples, Italy

Glucagon-Like Peptide-1 and Its Implications in Obesity

Veronica Hurtado, Isabel Roncero,
Enrique Blazquez, Elvira Alvarez and Carmen Sanz

Additional information is available at the end of the chapter

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1. Introduction

Glucagon-like peptide (GLP-1) is derived from the processing of the proglucagon gene. This peptide has diverse biological activities affecting peripheral tissues and the central nervous system. Thus, for example, GLP-1 stimulates pancreas insulin secretion in a glucose-dependent manner after eating, hence its denomination as an “incretin”. GLP-1 has also been considered an anorexigenic peptide, while also reducing cerebral glucose metabolism in the human hypothalamus and brain stem. These GLP-1 actions in the pancreas and central nervous system are achieved through GLP-1 receptors (GLP-1R) that share the same gene sequence in both tissues. In short, GLP-1 is an antidiabetogenic agent due to its action in the pancreas while acting in hypothalamic areas, helping to generate a state of satiety. Interestingly, GLP-1/exendin-4 administration in obese Zucker rats, which also develop insulin resistance, hyperinsulinemia and hyperlipidemia, reduces food intake and induced weight loss, which applies to lean rats, too.

The mid 20th century recorded the first indications that the hypothalamus plays a major role in feeding behaviour and energy homeostasis, whereby the electrical stimulation of the ventromedial hypothalamus (VMH) suppresses food intake, and the bilateral lesions of these structures induce hyperphagia and obesity. The VMH was therefore called the satiety centre. In contrast, alterations in the lateral hypothalamic area (LH) induced the opposite set of responses, and the LH was hence called the hunger centre. At least two kinds of glucose sensor neurons have been described in the brain: glucose-excited neurons are located mainly in the VMH and are excited by increased glucose levels in the extracellular space, while glucose-inhibited neurons (mainly present in the LH) are excited by decreases in glucose concentrations. A direct relationship has also been established between the regulation of food intake and energy homeostasis and hypothalamic metabolic sensor activities.

Both AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) and its downstream target p70 ribosomal protein S6 Kinase 1 (S6K1) contribute to detecting cellular energy and integrate nutrient and hormonal signals in order to maintain energy homeostasis in the organism. Thus, the Ser/Thr kinase AMPK is activated during energy depletion, when the AMP/ATP ratio increases and triggers a large number of downstream effectors by stimulating ATP-generating catabolic pathways and inhibiting anabolic pathways in order to restore the energy balance. Specifically, it has been reported that fasting increases, and re-feeding decreases, AMPK activity in several hypothalamic areas. Likewise, the hypothalamic mTOR/S6K1 pathway has also been involved in the control of feeding and in the regulation of energy balances. Thus, mTOR is activated by glucose and amino acids and, therefore, hypothalamic AMPK and mTOR/S6K1 respond to changes in glucose and other nutrients in the opposite way, and their effects on the regulation of food intake may overlap. Our recent results indicate that AMPK and S6K1 are functionally expressed in the VMH and LH areas, with differential activation in response to glucose fluctuations, in both in vitro models of hypothalamic organotypic slice cultures and animals in response to fasting and re-feeding, as well as in Zucker obese rats with a lower activation degree of hypothalamic AMPK in response to fasting.

In addition, we have reported that GLP-1/exendin-4 treatment inhibits the activities of AMPK and S6K1 when the activation of these protein kinases peak in both the VMH and LH areas. In pathophysiological situations, as occurs in Zucker obese rats, exendin-4 seems to act as a compensator for the variations in AMPK activity produced either by oscillations in glucose levels or by pathologies such as obesity or episodes of hyperinsulinemia.

In conclusion, it seems that GLP-1/exendin-4 acts in the VMH and LH, modulating the activation status of AMPK and S6K1 in response to glucose fluctuations, helping to improve pathophysiological states such as obesity and insulin resistance. The effects of these peptides in the hypothalamus are mediated through the activation of PKA, PKC and PI3K, as well as the phosphatase PP2.

2. Glucagon-like peptide-1: Dual role as an incretin and anorexigenic peptide

Glucagon and related peptides constitute a family derived from the proglucagon molecule, which is identical in sequence in the pancreas, intestine and brain [1], although post-translational processing of the precursor yields different products in these organs [2]. (Figure 1)

In gut L-cells, the C-terminal portion of proglucagon is predominantly processed to glucagon-like peptide-1 (GLP-1) and GLP-2. Further processing of GLP-1 in these cells produces the amidated and truncated forms of the peptide: GLP-1 [7-36] amide, GLP-1 [7-37] and GLP-1 [1-36] amide, with the first two being the biologically active forms, which are cited in the rest of the text as GLP-1. Although the truncated forms of GLP-1 are reported to have strong incretin activity, it is currently known that they are also important in the functioning of other peripheral tissues and the central nervous system. Both forms of the peptide are in-

distinguishable in their ability to produce biological effects through GLP-1 receptors located in pancreatic cells [3], gastric glands [4] and in adipocytes [5], lung [6] and brain [7-10].

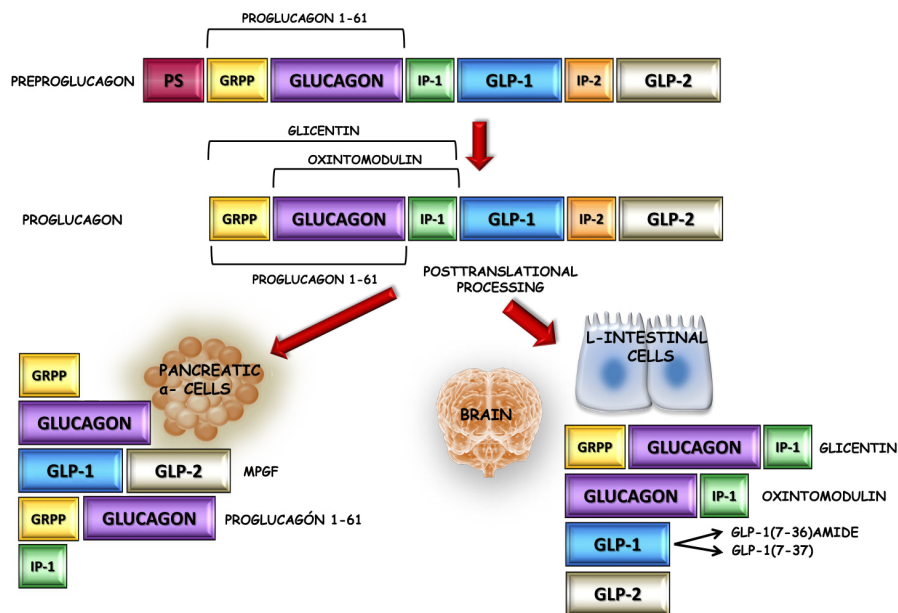


Figure 1. Posttranslational processing of preproglucagon. GLP: Glucagon-like peptide; GRPP: Glicentin-related pancreatic peptide; IP: Intermediate peptide; MPGF: the major proglucagon fragment; PS: Signal peptide.

In addition, GLP-1 and its own receptors are synthesized in the same brain regions, strongly supporting the actions of this peptide on the CNS. Thus, the perfusion of several brain nuclei with GLP-1 produces a selective release of neurotransmitters [11, 12], and the central and peripheral administration of this peptide inhibits food and drink intake [13-15]. The co-expression of GLP-1R, glucokinase, and glucose transporter protein 2 (GLUT-2) in the neurons involved in the control of food intake suggests that these cells may play a role in glucose sensing in the brain [14, 16-19]. Furthermore, GLP-1 has beneficial cardiovascular effects in humans by lowering blood pressure and improving myocardial function [20, 21], although in rats this peptide significantly increases arterial blood pressure and heart rate [22, 23]. Interestingly, GLP-1 has proliferative and antiapoptotic actions on pancreatic β -cells [24, 25], and has neurotrophic and neuroprotective features [21]. Considering the functions of GLP-1, its exendin-4 analogue is used in the treatment of type 2 diabetes [26].

Within the multiple functions of GLP-1, we have selected two important ones, namely, an incretin and an anorexigenic peptide.

2.1. GLP-1 actions as incretin hormone

The proposals made in 1906 by Moore et al. [27] on the antidiabetogenic effect of intestinal factors, and in 1929 by Zung & La Barre [28] on the release of a substance from intestinal mucosa with properties to decrease glycaemia, signalled the start of the development of the incretin concept and the study of the relationships between the gut and the endocrine pancreas. However, for many years these suggestions were ignored, until the development of radioimmunoassays, when Elrich et al. [29] demonstrated that the insulin secretion response to an oral glucose overload was greater to that obtained after intravenous perfusion with the same amount of glucose. This lends support to the belief that substances from the intestine were involved in the postprandial control of insulin secretion, which was referred to accordingly as the incretin effect. It is accepted that 20% to 60% of the increase in postprandial insulin secretion is due to this effect; with the broad oscillation being explained by the amount and composition of food intake.

The functional relationships between the intestine and the pancreatic islet were named by Unger and Eisentraut in 1969 [30] as the enteroinsular axis, while the criteria formulated by Creutzfeldt [31] considered a molecule to be incretin when it is secreted in response to nutrients, and that physiological concentrations increased the secretion of insulin in the presence of high glucose concentrations.

The first peptide described with incretin activity was the gastric inhibitory polypeptide (GIP) that went on to be referred to also as the glucose-dependent insulinotropic polypeptide (GIP). Thereafter, the observation of incretin activity after the inactivation of GIP suggested the existence of other molecules with an incretin effect. Thus, in experimental models where GIP was blocked by its own antibodies 50-80% of incretin activity was still observed. We now know that GLP-1 has a greater incretin effect than GIP, being considered the most powerful incretin molecule of all those known. In other words, a molecule with incretin activity may be defined as a hormone of intestinal origin that potentiates the secretion of insulin after the oral ingestion of nutrients. Knowledge of incretins has been very useful for a better understanding of certain pathophysiological entities [32]. In 1986, Nauck et al. [33] first documented a reduced incretin effect in patients, with type 2 diabetes. It is important to note that Nauck et al. described this reduced effect with GIP and not with GLP-1, because at that time GIP was the only incretin known. However, a year later [34], GLP-1 was identified as an incretin hormone and shown to be more effective than GIP to stimulate insulin secretion on a molar basis and at an equivalent level of glucose concentration [35]. Both in non-diabetic and type 2 diabetic subjects, GLP-1 was more effective than GIP at enhancing insulin secretion and lowering glucagon concentrations [36].

The recognition that native GLP-1 is quickly degraded by the protease dipeptidyl peptidase IV (DPP-4) led to the development of GLP-1 agonists that are resistant to this enzyme [37]. The degradation by DPP-4 of exenatide and liraglutide and DPP-4 inhibitors (sitagliptin, saxagliptin, vildagliptin and linagliptin) currently represents an effective therapeutic option for patients with type 2 diabetes. Furthermore, several agents have been developed in recent years, including longer acting DPP-4 resistant GLP-1 agonists.

In addition, many biological effects of GLP-1, other than incretin actions, have been reported in recent decades, representing a good tool for several therapeutic treatments. These GLP-1 effects include properties such as an anorexic peptide, beneficial cardiovascular actions in humans, increased pulmonary surfactant formation in human and experimental animals, pancreatic islet neogenesis and proliferative and antiapoptotic actions. GLP-1 receptors are also widely expressed in the brain [9, 10], where their agonists produce a selective release of neurotransmitters [11, 12] and increase GLP-1 receptor expression in glia after a mechanical lesion of the rat brain has been reported [38]. Accordingly pre-clinical data suggest a neuro-protective/neurotrophic function of GLP-1, and some authors have proposed that this peptide may have a positive potential role for reversing neurodegenerative disorders [21].

2.2. GLP-1 actions in the control of food intake

A number of peptide hormones, previously thought to be specific to the gastroenteropancreatic system and later found also in the mammalian brain, have been shown to modulate appetite, energy homeostasis and body weight. They have these physiological effects together with other neuropeptides, such as neuropeptide Y (NPY), opioid peptides, galanin, vasopressin, and GHRH. Peptide Y (Y_{3-36}) is also released from the gastrointestinal tract postprandially, and acts on the NPY Y_2 receptor in the arcuate nucleus to inhibit feeding, with a long-term effect [39]. Conversely, other satiety signals induced by gut-brain peptides such as GLP-1 [13-15], GLP-2 [40] and cholecystokinin produced a short-term effect, while insulin and leptin [41] inhibit the appetite by increasing the formation of pro-opiomelanocortin (POMC) and reducing NPY action. In addition, ghrelin, a peptide released by the stomach, is stimulated before meals to facilitate NPY action.

GLP-1 and GLP-2 significantly modify feeding behaviour. The intracerebroventricular (icv) or subcutaneous administration (sc) of GLP-1 produced a marked reduction in food intake and water ingestion [13-15]. Exendin-4 proved also to be a potent agonist of GLP-1 by decreasing both food and water intake in a dose-dependent manner. Pre-treatment with exendin [9-39], an inhibitor of the GLP-1 receptor, reversed the inhibitory effects of GLP-1 and exendin-4. These findings suggest that GLP-1 may modulate both food and water intake through either a central or peripheral mechanism. Similar results have been found in humans when the peptide was administered in the periphery [42]. After the subcutaneous administration of GLP-1, it could enter the brain by binding to blood-brain-barrier-free organs such as the subfornical organ and the area postrema [43], or through the choroid plexus, which has a high density of GLP-1 receptors [17].

Several observations suggest a possible action of GLP-1 on thirst-regulatory mechanisms, since GLP-1R mRNA has been located in brain areas related to the control of thirst, such as the preoptic area, glial cells lining the third ventricle and, especially, the neurons of the PVN, which is a key station for water balance regulation through the antidiuretic effects of vasopressin released by its projection to the neurohypophysis [44]. In addition, the icv administration of GLP-1 significantly increases the circulating levels of vasopressin, and the colocalization of the mRNA of the GLP-1 receptor, and vasopressin has been found in the neurons of the PVN [45].

The control of feeding behaviour by GLP-1 and exendin-4 has been explored in Zucker obese rats, resulting in a reduction in food intake, with exendin-4 being much more potent than GLP-1. The long-term sc administration of exendin-4 decreased daily food intake and practically blocked weight gain in obese rats. These observations highlight the potential usefulness of exendin-4 as a tool for treating obesity and/or diabetes. Both GLP-1 and exendin-4 control blood glucose through the stimulation of glucose-dependent insulin secretion, the inhibition of glucagon secretion, and delayed gastric emptying [34, 46, 47], which facilitate the decrease in blood glucose in type 1 and type 2 diabetic patients [48]. In the light of these results, different N-terminal substituted GLP-1 analogues resistant to DPP-IV have recently been developed. These resistant analogues have a prolonged metabolic stability in vivo and improved biological activity, which is of great interest in the treatment of type 2 diabetes and/or obesity.

On the other hand, the icv administration of GLP-2 to mice and rats produced a marked decrease in food intake but not in water ingestion [43]. Surprisingly, this effect was avoided by the administration of exendin [9-39], an antagonist of GLP-1.

3. Importance of the VMH and LH in the control of food intake

In recent years, researchers have been focusing on the relationship between gut hormones and the brain areas controlling appetite, ingestion, food reward and body weight [49, 50].

Both gut and brain are considered the main organs responsible for controlling body weight. The hypothalamus is the focus of many of the peripheral signals and neural pathways that control energy homeostasis and body weight. However, new evidence has been forthcoming in recent years to suggest that human food intake is also controlled by other areas in the central nervous system, such as subcortical and cortical areas.

The hypothalamus regulates body weight by precisely balancing the intake of food, energy expenditure and body fat tissue. The role of the hypothalamus in regulating food intake and body weight was established in 1940 [51] through the classical experiments by Hetherington and Ranson. They placed bilateral electrolytic lesions in a vast region of the hypothalamus, occupied by the dorsomedial and ventromedial areas, the arcuate nucleus, the fornix and a portion of the lateral hypothalamic area (without disturbing the pituitary gland). The results were a marked adiposity characterized by a doubling of body weight and a huge increase in body lipids. A few years later, Anand and Brobeck [52] continued these experiments in greater detail, demonstrating that lesions of the lateral hypothalamus at the level adjacent to the ventromedial nucleus caused loss of appetite, inanition, and even death by starvation. Thus, the lateral hypothalamic area acted as a “feeding centre” and the ventromedial nucleus as a “satiety centre”. Since then, it has been established that the “dual centre model” regulates feeding [53], that the proposed lesioning of the VMH increases appetite, while stimulating the VMH decreases it. By contrast, lesioning or stimulating the LH decreases or induces appetite, respectively.

The dual control theory of feeding is based on the homeostatic view of hunger and satiety, together with the consideration of glucose not only as a metabolic fuel, but also as a signaling molecule, and the existence of specialized neurons containing glucose sensors that activate or inhibit feeding when blood glucose levels change. A fall in glucose would activate the LH and, consequently, give rise to hunger. Hunger leads to the consumption of food and thus to an elevation of glucose levels that would activate the VMH, leading to a feeling of satiety that will stop the feeding, and eventually glucose levels would fall again.

Over the last 25 years, there has been a dramatic increase in studies on the hypothalamus. Knowledge of the hypothalamus has only recently evolved from anatomical concepts (nuclei, 'areas' and fibre tracts) to neurochemicals (characterizing the distributions of neuropeptides and transmitters and their receptors), focusing on the modulation of feeding behaviour and energy expenditure. We are now beginning to reach the stage of functionally understanding the molecular mechanisms, defining exactly which neuronal populations respond to specific nutritional and related signals, and how they pass on that information. At least 25 transmitters have been suggested to play key roles in feeding behaviour [54]. Accordingly, the dorsomedial and paraventricular regions are important within the hypothalamus, along with the well-established VMH and LH [55]. The VMH, which consists of the ventromedial and arcuate nuclei, respectively, is a key region for integrating the peripheral signals of nutrient status and adiposity. The arcuate nucleus contains neurons with NPY/AgRP and POMC, which have opposing effects on energy homeostasis. Thus, NPY increases food intake and activates energy sparing mechanisms, while melanocortins decrease food intake and increase energy expenditure [56]. On the other hand, the LH, the classical "feeding centre", is a heterogeneous area that receives a multitude of neuronal inputs from many areas known to be important in the regulation of energy homeostasis: LH encompassing neurons and terminals containing orexigenic peptides. Basically, the LH contains two distinct neuronal cell populations that regulate feeding behaviour: containing hypocretin/orexin [57, 58] and a melanin-concentrating hormone (MCH) [59] (Figure 2).

In addition to its response to circulating peptides and hormones that reflect energy status, the brain, and specifically the hypothalamus, also senses and responds to changes in blood glucose levels [14, 16, 18, 19, 60]. There are several areas in the brain acting as a glucose sensor. Examples of these are the hypothalamus [60, 61], nucleus solitarius [62] and amygdala [63]. The glucose sensing neurons located in these areas monitor energy status and initiate the responses to maintain glucose and energy homeostasis. Besides the brain glucose sensors, there are also glucose sensors located in peripheral tissues including the intestine [64], the carotid body [65] and mesenteric veins [66]. In fact, glucose sensors in the hypothalamus were first discovered in the VMH and LH [57, 58]. Moreover, interstitial glucose levels in the VMH and LH vary with blood glucose concentration [67], and these changes in glucose have been postulated to trigger meal initiation. Since glucose is the brain's primary fuel, it should respond to a severe glucose deficiency. In this way, VMH glucose sensors may play a role in detecting and countering severe glucose deficiency [68]. However, Levin et al. recently showed there was no correlation between VMH glucose levels and spontaneous feeding

[69]. It is therefore unlikely that VMH glucose sensors regulate meal-to-meal food intake, although it does not rule out a role for glucose sensors in the LH or other brain regions.

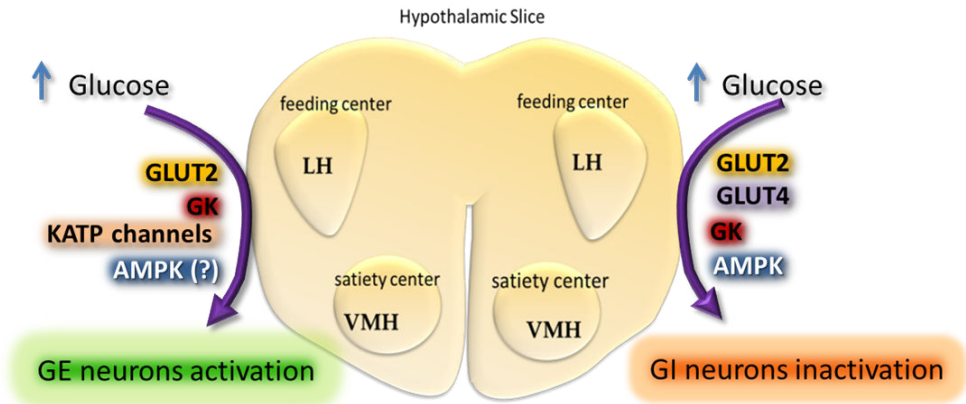


Figure 2. Schematic representation of a hypothalamic slice. Localization of the VMH and LH are indicated. GE and GI neurons are activated or inactivated by a rise in glucose, respectively. The putative components responsible for glucose sensing in GE and GI are shown.

Glucose sensing neurons are those that alter their frequency of potential actions in response to changes in interstitial glucose levels [60, 70]. There are mainly two neurons whose activity is regulated by alterations in glucose levels [60]: Glucose-excited (GE) neurons that increase their potential frequency in response to increases in interstitial glucose from 0.1 to 2.5 mM glucose. The other kind of neurons (GI) are those that decrease their frequency of potential actions when glucose rises. More recently [71], other neurons have been described that respond to an increase of more than 5 mM glucose. Thus, high GE (HGE) and high GI (HGI) neurons increase or decrease their frequency of potential actions, respectively, in response to increases in interstitial glucose from 5 to 20 mM, although these neurons are still not thoroughly characterized, and there are doubts about their physiological significance. However, it is important to consider that the interstitial brain glucose concentration is approximately 30% of the concentrations found in the blood. Thus, when the peripheral plasma glucose concentration is 7.6 mM, the interstitial VMH glucose is only 2.5 mM [67]. Decreasing plasma glucose to 2–3 mM or increasing to 15 mM resulted in brain glucose levels of 0.16 mM and 4.5 mM, respectively [67, 72]. Therefore, glucose concentrations found within the majority of the brain in vivo under physiological and pathophysiological conditions are within the 0.2 to 5 mM range [73–76], and it seems that the GE and GI neurons are mainly responsible for glucose sensing, since it is unclear whether brain glucose levels ever exceed 5 mM in the presence of an intact blood brain barrier. However, it should be noted that hyperglycemia impairs the integrity of the blood brain barrier [77], raising the question of whether HGE and HGI neurons could have a physiological significance in hyperglycemia-associated pathology. Nevertheless, it seems that glucose sensing neurons could be functioning to protect the brain against a severe energy deficit.

The components responsible for glucose sensing in GE neurons seem to be shared with those present in pancreatic beta-cells: GLUT2, as well as glucokinase. Furthermore, GE uses the ATP-sensitive potassium (KATP) channel to sense glucose, as occurs in beta-cells. However, while KATP channels are expressed in all GE neurons, only approximately half of VMH GE neurons express glucokinase, and approximately 30% express GLUT2 [78].

Multiple subtypes of GE neurons may exist that use alternate glucose sensing strategies. Thus, Claret et al. have shown that transgenic mice lacking the $\alpha 2$ subunit of AMPK, an important cellular fuel gauge, also lack GE neurons in ARC [79]. However, other authors [80] have shown that the acute pharmacological activation or inhibition of AMPK had no effect on glucose sensing in VMH GE neurons.

GI neurons have similar components to GE and, therefore, to beta-cells in glucose sensing, such as glucokinase [78, 81], as well as GLUT2 and GLUT4. However, the signal transduction pathway, whereby changes in intracellular ATP alter the activity of GI neurons, is completely different. In this case, the activation of $\alpha 2$ AMPK by glucose mediates the activation of VMH GI neurons, as described by Murphy et al. [82]. Hypothalamic $\alpha 2$ AMPK is a key kinase involved in the energy balance and is a target for a number of hormones and a transmitter that regulates the energy balance [83-88]. The pharmacological activation of hypothalamic AMPK increases food intake [89]. It is not therefore surprising that decreased glucose activates AMPK in GI neurons.

4. AMPK, together with mTOR and its downstream target S6K1, integrate nutrient and hormonal signals to maintain energy homeostasis

AMPK is a nutrient and energy sensor. AMPK senses cellular energy availability by detecting the AMP/ATP ratio. AMPK is activated in low energy states and promotes ATP-generating catabolic pathways and inhibits anabolic reactions [90-92].

AMPK is a heterotrimeric complex that contains a catalytic α -subunit ($\alpha 1$ or $\alpha 2$) and two regulatory subunits, β ($\beta 1$, $\beta 2$) and γ ($\gamma 1$, $\gamma 2$, $\gamma 3$). The α -subunit contains a kinase domain. The β -subunit contains the regions that permit interaction with other α and γ subunits and a carbohydrate-binding domain that facilitates binding to glycogen. The γ subunit contains four tandem repeats, which are four binding sites for adenosine derivatives denominated as CBS motifs (cystathionine β -synthase) [92, 93].

Different isoforms and the alternative splicing of some mRNAs encoding these subunits give rise to a wide range of heterotrimeric combinations. The expression of the catalytic subunits ($\alpha 1$ and $\alpha 2$) is also different. The $\alpha 2$ subunit expression has been found in pancreatic beta-cells, neurons, skeletal muscle and the heart. The liver has 50% of each AMPK α isoform ($\alpha 1$ and $\alpha 2$), while adipose tissue expresses higher levels of the AMPK $\alpha 1$ isoform [93].

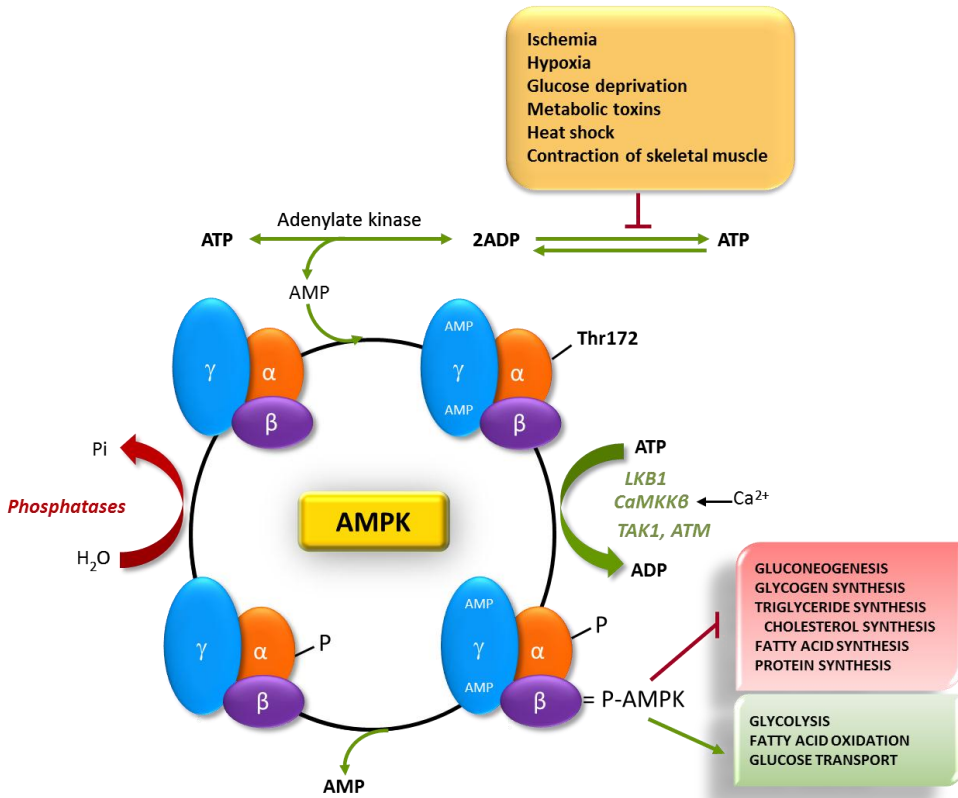


Figure 3. Schematic representation of AMPK’s subunits and its activation process. AMPK detects changes in the cellular energy state that occur in response to nutrient variations or metabolic stress caused by changes in the AMP/ATP ratio. The activation of AMPK triggers key enzymes of glucose metabolism and fatty acids. The long-term effect of AMPK activation is the transcriptional control of the main elements involved in these metabolic pathways.

AMPK serine/threonine kinase activity is stimulated by the phosphorylation of the α -subunit on the Thr residue (Thr172). This activation process is regulated by several upstream kinases. The two main kinases in mammals are liver kinase B1 (LKB1), identified as a tumour-suppressor, and the Ca^{2+} /calmodulin-dependent protein kinase (mainly CaMKK β) [92, 93]. AMPK activity is also allosterically regulated by AMP binding to the γ subunit (Figure 3). Recent studies have found that AMP or ADP binding to the γ regulatory subunit protect the activated phosphorylated form of AMPK [94, 95]. AMP, ADP and ATP bind the γ subunit with similar affinity [94]. AMPK can be activated by increases in AMP and ADP according to changes in the cellular levels of adenosine derivatives. LKB1 phosphorylates AMPK in almost all tissues, while CaMKK β plays an important role in neurons and T lymphocytes. Other studies have suggested that a member of the MAPKKK family, TAK1 (transforming growth factor β -activated kinase) could be an important AMPK upstream kinase in cardiac

cells [96], and ATM (ataxia telangiectasia mutated) may also regulate the phosphorylation of Thr172 [97]. The level of Thr172 phosphorylation depends also on the activity of protein phosphatases [98, 99]. The effect of an increase in AMP inhibits phosphatase activity, and considering that LKB1 is constitutively active [100], the response after a rise in AMP increases the phosphorylation of Thr172 and the activation of AMPK.

AMPK can detect changes in cellular energy state that occur in response to nutrient variations. Any cellular or metabolic stress that reduces ATP production (e.g., heat shock, hypoxia, ischemia, glucose deprivation) or accelerates ATP consumption (e.g., contraction of skeletal muscle) will increase the ADP/ATP ratio, which will be amplified by the action of adenylate kinase, resulting in increased AMP/ATP with the consequent activation of AMPK (Figure 3). Once activated, AMPK first directly affects the activity of key enzymes of glucose metabolism and fatty acids, and second, proceeds to the long-term regulation of the transcriptional control of the main elements involved in these metabolic pathways. The net result of the activation of AMPK will restore the energy balance, inhibiting the anabolic pathways responsible for the synthesis of macromolecules, such as proteins and glycogen, and also of the following lipids: fatty acids, triglycerides and cholesterol, while activating the catabolic pathways, such as the oxidation of fatty acids, glucose uptake and glycolysis [101] (Figure 3).

The mTOR is a serine/threonine kinase that responds to nutrients and hormonal signals [102-104]. mTOR forms two distinct complexes with different sensitivities to rapamycin: mTORC1 and mTORC2. Both complexes contain mTOR, GβL (G-protein β-protein subunit-like), mLST8 (mammalian lethal with SEC13 protein) and deptor (DEP domain containing mTOR interacting protein) [103, 105, 106]. This complex, along with raptor (rapamycin-sensitive adaptor protein of mTOR) and PRAS40 (proline-rich Akt substrate of 40 kDa) forms mTORC1, which is rapamycin and nutrient sensitive. However, mTORC2 comprises mTOR, GβL/mLST8 and deptor together with rictor (rapamycin-insensitive companion of mTOR), mSin 1 (mammalian stress-activated MAPK-interacting protein 1) and protor 1/2 (protein observed with rictor 1/2), which is insensitive to acute rapamycin (Figure 4)

mTORC1 regulates metabolism and cell growth in response to several environmental signals. The presence of amino acids, growth factors and mitogens stimulates mTORC1, which promotes anabolic processes. The mTORC1 activity phosphorylates multiple substrates, with S6K1 and the initiation factor 4E binding proteins (4E-BPs) being the best characterized [106-108]. The activation of mTORC1 induces the dissociation of 4E-BP from the eukaryotic translation initiation factor 4E (eIF4E), facilitating mRNA translation [109], and the activation of S6K1 promotes protein synthesis. Moreover, the mTORC2 function is less well known. It is known that mTORC2 phosphorylates Akt and appears to regulate mainly cell proliferation and cell survival [110] (Figure 4)

The effect growth factors have on mTORC1 is mediated through phosphatidylinositol-3,4,5-triphosphate kinase (PI3K) activation, the subsequent activation of phosphoinositide-dependent kinase (PDK1) and Akt. Once activated, Akt phosphorylates tuberous sclerosis complex 2 (TSC2), suppressing the inhibitory effect of the TSC1-TSC2 complex in mTORC1.

TSC2 functions as a GTPase-activating protein of Ras homolog enriched in brain (Rheb), which is an mTORC1 activator. Mitogens activating the Ras/MAPK cascade also activate mTORC1. ERK phosphorylates TSC2, inhibiting the TSC1/TSC2 complex and inducing mTORC1 activity [111]. Raptor is additionally phosphorylated by ERK [112] (Figure 4)

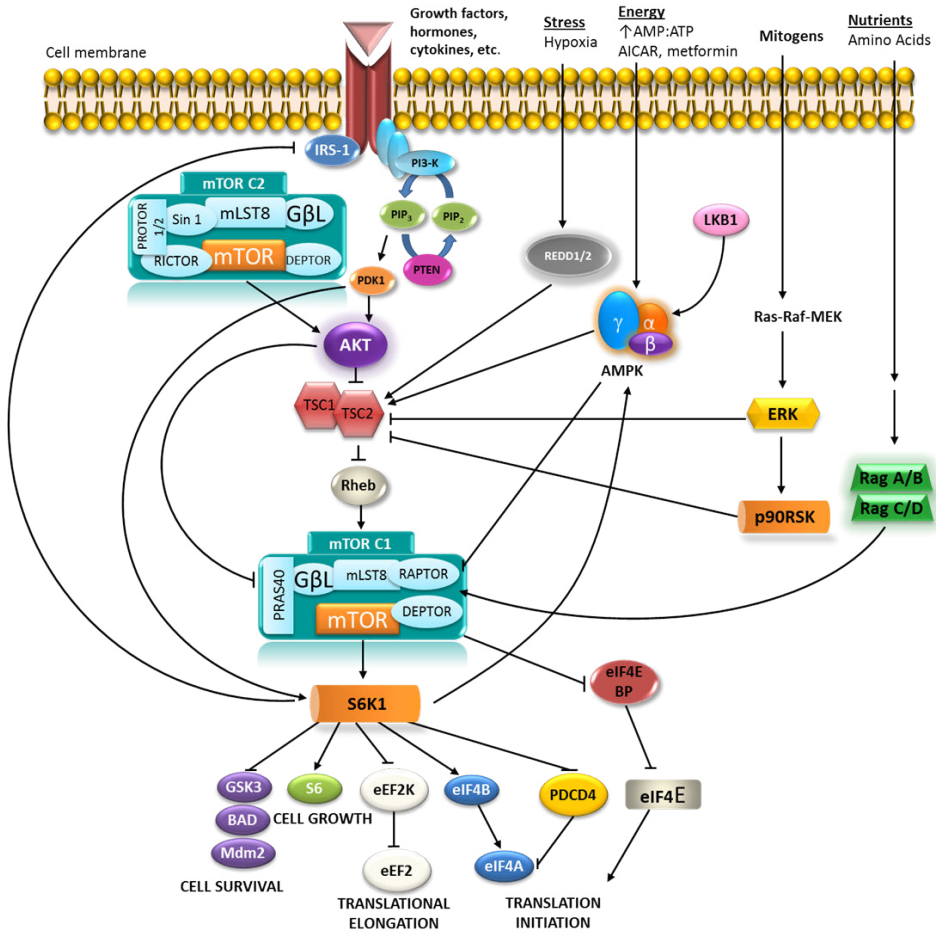


Figure 4. Network of proteins involved in the AMPK/mTOR/S6K1 signalling pathway

mTORC1 is also stimulated by amino acids, especially leucine. This activation pathway is independent of PI3K. The amino-acid regulation of mTOR needs rag GTPases and Rheb. The detailed mechanism of activation is unknown. It has been suggested that rag GTPases may control the localization of mTOR to specific vesicular membranes containing Rheb-GTP [113].

mTORC1 activity is inhibited in conditions of energy depletion coordinated with AMPK activity. An increase in AMP/ATP ratio activates AMPK, which phosphorylates the TSC2, and this modification induces the concomitant inhibition of mTORC1 mediated by the TSC1-TSC2 complex [114]. Furthermore, AMPK phosphorylates raptor in mTORC1, which down-regulates this complex [115].

In addition, energy depletion inhibits mTORC1 by a mechanism that is independent of AMPK activation. This effect is mediated by eliminating the GTP loading of Rheb [116].

4.1. Expression and regulation of these sensors in the VMH and LH

Studies in recent years have established a direct relationship between metabolic sensor activity in the hypothalamus and the regulation of food intake, body weight and energy homeostasis.

AMPK is broadly expressed throughout the brain. The $\alpha 2$ catalytic subunit is present with a high distribution in neurons and activated astrocytes [117]. Hypothalamic AMPK has been assumed to play a role in the central regulation of food intake and energy balance, whereby fasting increases and re-feeding decreases AMPK activity in various hypothalamic nuclei. Alterations of hypothalamic AMPK activity specifically affected $\alpha 2$ AMPK and did not change $\alpha 1$ AMPK [88].

The hypothalamic AMPK role has been studied *in vivo* by the expression of AMPK mutants: dominant negative AMPK (DN-AMPK) or constitutively active AMPK (CA-AMPK). The expression of CA-AMPK in the medial hypothalamus by adenoviruses increased food intake and body weight, whereas the expression of DN-AMPK inhibited them [88]. These alterations change the hypothalamic neuropeptide expression: the expression of CA-AMPK enhances the effect of fasting, increasing the expression of NPY and AgRP in the ARC and the melanin-concentrating hormone in the lateral hypothalamus, whereas the hypothalamic expression of DN-AMPK decreases the expression of orexigenic neuropeptides NPY and AgRP in ARC [88, 118].

mTORC1 is another metabolic sensor that plays an important role in the regulation of feeding behaviour and body weight in the hypothalamus [119, 120]. mTOR and its downstream target S6K1 are widely distributed in the rat brain. The activated forms of mTOR and S6K1 are localized mainly in the paraventricular and arcuate nuclei [119], being co-localized in a high percentage of orexigenic neurons that express AgRP/NPY and also in around half the anorexigenic neurons that express POMC/CART in the arcuate nuclei [119].

mTORC1 activation in the rat hypothalamus decreases food intake and body weight. Similar results were found by introducing constitutively active S6K1 mediated by adenovirus into the mediobasal hypothalamus of the rat brain. By contrast, the injection of dominant-negative S6K1 leads to an increase in food intake and body weight [121].

4.1.1. Nutrient regulation

Food intake leads to periods of fasting and feeding that are associated with substantial changes in the level of available nutrients (e.g., glucose and amino acids) and accompanied

by hormonal changes. Several studies describe the effect of glucose on both metabolic sensors, AMPK and mTOR, in hypothalamic areas. During fasting, the decrease in glucose concentration activates AMPK. This period is also characterized by low levels of glucose and amino acids, and the mTOR complex is kept inactive. The increase in glucose levels after food intake decreases the activity of AMPK and, conversely, the activity of the mTOR/S6K1 pathway is stimulated by higher levels of glucose and amino acids.

Thus, Kim et al. reported that decreasing intracellular glucose through the supply of 2-deoxyglucose increases hypothalamic AMPK activity and food intake. By contrast, hyperglycaemia decreases hypothalamic AMPK activity [122]. It was also observed that AMPK activity is inhibited in arcuate, ventromedial, dorsomedial, paraventricular nuclei and the LH by high glucose and re-feeding. [88]. Increases in α 2-AMPK activities in arcuate-ventromedial and paraventricular nuclei are also detected during insulin-induced hypoglycaemic in rats [123]. However, fasted rats recorded a decrease in the number of hypothalamic cells expressing mTOR and S6K1 activated forms specifically in the arcuate nucleus, with these changes responding to the availability of nutrients [119]. Similar findings were subsequently confirmed, also showing that the constitutive activation of S6K in the mediobasal hypothalamic area protects against the harmful effects of a high-fat diet [121].

It has also been established that AMPK and mTOR are involved in the anorexigenic effect induced by high protein diets. Thus, a high protein diet and the intracerebroventricular administration of leucine decreased AMPK phosphorylation in the rat hypothalamus [120]. The activation of hypothalamic mTORC1 is additionally produced by a high protein diet and the intracerebroventricular administration of amino acids or leucine [119, 124].

4.1.2. Gut hormone regulation and signals from energy stores

The effects of glucose, amino acids and other nutrients are reinforced by the effects of intestinal peptides, as stated above. They are able to regulate food intake, energetic homeostasis and body weight. The gastrointestinal tract responds to gut contents by secreting hormones, which can serve to inform the CNS of nutrient status. Thus, circulating ghrelin, the only intestinal peptide with orexigenic properties, is high in the period before a meal, and the level declines an hour after eating [125]. Ghrelin stimulates food intake in lean and obese humans [126]. In contrast, anorexigenic intestinal peptides as peptide YY (PYY), pancreatic polypeptide (PP), GLP-1, oxyntomodulin and cholecystokinin are low during the fasting period, and their level increases after a meal, and some of them are released proportionally to the amount of calories ingested (Reviewed in [127]).

Other signals that inform the state of energy stores, such as leptin and insulin levels, are important modulators of feeding behaviour. Insulin regulates the storage of nutrients and also informs the brain about the energy balance [128]. Leptin is produced by adipose tissue and informs the brain about the energy storage status [128].

We now know that the function of at least some of these peptides may be mediated by the modulation of hypothalamic metabolic sensors. Thus, it has been reported that hypothalamic AMPK activity is also regulated by several orexigenic and anorexigenic signals. Ghrelin,

the intestinal peptide with orexigenic properties, activates AMPK and stimulates food intake [87, 89]. By contrast, anorexigenic peptides such as leptin decrease AMPK activity in the ARC and PVN [88, 89, 129]. However, leptin treatment increased mTOR and S6K1 hypothalamic activity [130].

It has recently been suggested that ghrelin activates AMPK in presynaptic neurons, inducing an increase in activity in NPY/AgRP neurons promoting sustained food intake, and this signal stops after leptin is released by adipose tissue, signalling to stimulate POMC neurons inhibiting feeding and also to inhibit the AMPK in the presynaptic neurons, inactivating the release of NPY/AgRP [131].

Inoki et al. previously reported that the activation of AMPK induces the inhibition of mTOR activity [114]. It has recently been posited that S6K phosphorylates $\alpha 2$ AMPK. This process is necessary for the leptin effects on hypothalamic AMPK activity [132].

These findings indicate that hypothalamic AMPK and mTOR respond to changes in glucose and other nutrients in opposite ways, and their effects on the regulation of food intake may overlap.

5. Modulation of AMPK and S6K by GLP-1/exendin-4 in these hypothalamic areas

As indicated before, GLP-1 is able to induce several effects contributing to the control of feeding behaviour. It inhibited gastric acid secretion and emptying, stimulated postprandial insulin secretion and inhibited glucagon release. GLP-1 treatment to type 2 diabetic subjects normalized the fasting levels of blood glucose and decreased postprandial glucose levels. We have also reported that GLP-1 reduces glucose metabolism in the human hypothalamus and brain stem [133].

In general, the brain activity of AMPK is activated by fasting and is inhibited by re-feeding [88, 122, 123], but the effect of glucose on AMPK also regulates *Ampk* expression in VMH [134]. Thus, fasting increased *Ampk* mRNA expression in the hypothalamus of rats, and the ICV administration of GLP-1 reduced that effect [135].

The glucose effect on AMPK might be region-specific in hypothalamic areas that have opposite effects over the control of feeding behaviour. We have also reported, using rat hypothalamic slices, that high glucose levels decrease the expression of *Ampk- $\alpha 2$* mRNA, specifically in the LH, but not in the VMH [83]. The decrease in *AMPK $\alpha 2$* expression in response to high glucose levels was reversed by the presence of GLP-1 [83]. Sanz et al. have also reported a different response to glucose in the VMH and LH [136, 137]. The distinctive response in the LH compared to the VMH may be explained by the different role these two areas have in the control of food intake.

Results obtained from in vivo studies conducted on lean and obese Zucker rats showed that the effects fasting and re-feeding have on the activity of AMPK and S6K in the areas involved in the control of feeding are modulated by exendin-4 treatment [83].

It has been previously reported that the anorexigenic effects produced by the intraperitoneal administration of exendin-4 led to a reduction in food intake and increased the period between meals [138]. Additionally, the peripheral administration of exendin-4 and liraglutide regulates food intake by activating the GLP-1 receptors expressed on both vagal afferents and CNS [139]. Recent studies conducted within our group [83] have focused on clarifying the coordinated effects of fasting, re-feeding and exendin-4 administration on the activity of AMPK and S6K in the VMH and LH. The results of these studies show that fasting increases hypothalamic AMPK activity in both areas in lean Zucker rats. However, the subcutaneous administration of exendin-4 over the last hour reversed this effect, whereas exendin-4 activated AMPK in animals re-fed for two hours when AMPK activity was markedly inhibited. The activation degree of AMPK after four hours of re-feeding differed in both areas. Thus, the activation level of AMPK in the VMH was similar to fasted rats. However, AMPK activity in the LH was still low, and exendin-4 treatment decreased AMPK activity in the VMH, whereas no significant effect was detected in the LH (Figure 5).

Anorexic peptides also regulate the mTOR/S6K1 pathway in hypothalamic areas. Insulin and leptin increases the activated forms of S6K [119]. The administration of exendin-4 also regulates S6K activity and the effect is dependent on the activation status of S6K, as occurred with AMPK. We thus found that S6K activation peaked in animals re-fed for four hours. However, the administration of exendin-4 strongly stimulated S6K activity in animals re-fed for two hours. In contrast, exendin-4 decreased S6K activity in the VMH of lean rats re-fed for four hours [83] (Figure 5).

The use of rat organotypic hypothalamic slices confirmed that AMPK activity at low glucose concentrations was stimulated, and S6K activity was maintained with minimal activation [83]. GLP-1 treatment reversed the effect of glucose on AMPK and did not modify S6K activity in the VMH and LH. High levels of glucose stimulated S6K activity in both nuclei, and the presence of GLP-1 reversed such activation. Similar results were found using hypothalamic GT1-7 and neuroblastoma N2A cell lines [83]. The metabolic sensors in these cells respond to glucose as described above, and GLP-1 treatment reversed the glucose effects [83].

The effect of GLP-1 on AMPK activity was also reported in other brain areas. Thus, GLP-1R activation in hindbrain suppressed food intake, and that effect is accompanied by the suppression of AMPK activity [140].

The complexities of the regulation of hypothalamic AMPK activity have previously been described for some hormones. Thus, the cocaine-and amphetamine-regulated transcript (CART) has been reported to have an anorexic effect after intracerebroventricular administration [141], while CART injected directly into the paraventricular or arcuate nucleus of fasted rats increases food intake [142]. Likewise, differences in the effect of regulatory peptides on AMPK as a function of nutritional status have been previously described. Ghrelin or cannabinoids have ad libitum effects [143], whereas leptin [88] and adiponectin [144] only have an effect after variable periods of fasting or re-feeding.

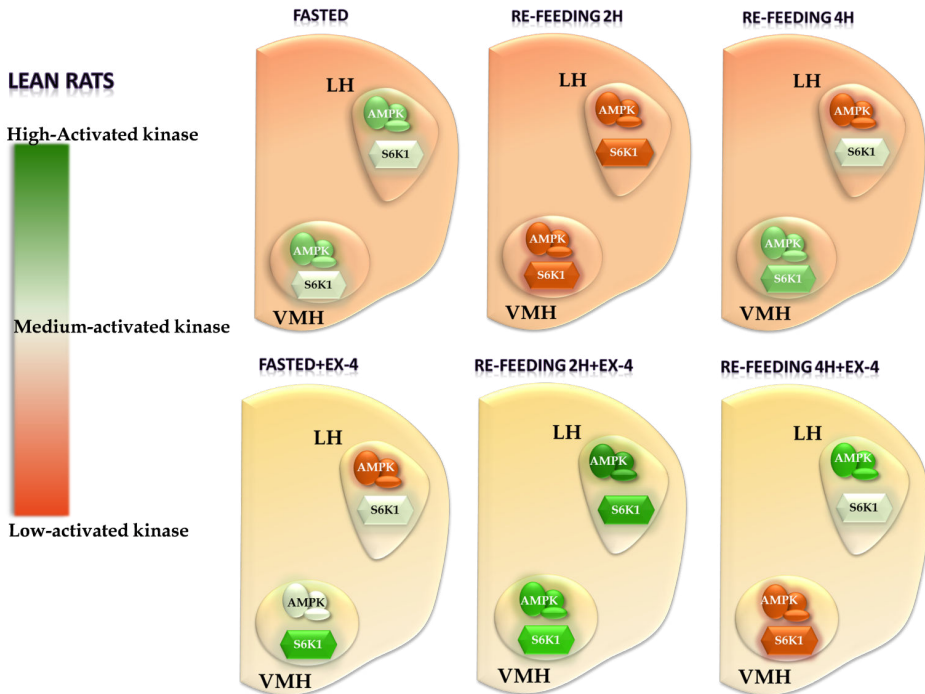


Figure 5. Effects of exendin-4 administration in fasted or re-fed lean rats on the activity of AMPK and S6K. Lean Zucker rats were fasted or re-fed for two or four hours. In some cases, the GLP-1 analogue exendin-4 (100 nM) was administered. The activation states of AMPK and S6K were determined by quantifying phospho-specific forms in VMH and LH areas.

6. GLP-1/exendin-4 as a compensator of the disturbances in AMPK and S6K activities occurring in obesity

In obesity, the elevated levels of nutrients and hormonal modifications alter the activity of hypothalamic metabolic sensors. Thus, diet-induced obesity reduced hypothalamic AMPK activity [145]. The GLP-1 receptor agonist exendin-4 is one of the agents used in the treatment of type 2 diabetes [146] and is a long-acting receptor agonist of GLP-1 that also produces weight loss [147-149].

The obese Zucker (*fa/fa*) rat provides a well-established animal model of insulin resistance and genetic obesity and, in comparison with lean Zucker rats, manifests hyperinsulinemia and hyperlipidemia. We have previously noted that the peripheral long-term subcutaneous administration of exendin-4 decreased food intake and induced weight loss in both obese and lean control Zucker rats [15].

Zucker rats have been used to analyze the exendin-4 effect on the activity of AMPK and S6K in the VMH and LH areas [83]. The results obtained showed that AMPK activity was lower in the obese than in the lean Zucker rats in both areas. Interestingly, the effect of exendin-4 administration on fasted obese Zucker rats was different compared to the lean rats. The absence of exendin-4 effect in obese rats maintains AMPK activity at a level of activation similar to the lean animals after the administration of exendin-4 [83] (Figure 5).

These results suggest that GLP-1/exendin-4 might compensate for the alterations in AMPK activity produced either by oscillations in glucose levels or by pathologies such as obesity or episodes of hyperinsulinemia (Figure 5, 6).

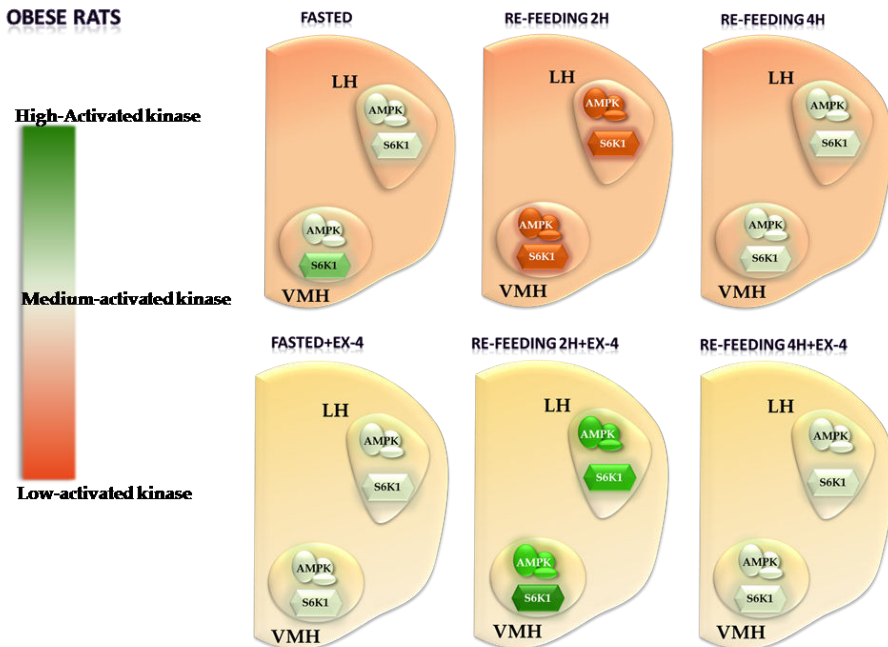


Figure 6. Effects of exendin-4 administration in fasted or re-fed obese rats on the activity of AMPK and S6K. Lean Zucker rats were fasted or re-fed for two or four hours. In some cases, the GLP-1 analogue exendin-4 (100 nM) was administered. The activation states of AMPK and S6K were determined by quantifying phospho-specific forms in the VMH and LH areas.

Another difference in the level of activation of AMPK between obese and lean Zucker rats was observed after re-feeding for four hours. The AMPK activity in the LH was higher in obese compared to lean animals (Figure 5, 6).

After two hours of re-feeding, exendin-4 treatment increased S6K activity in the VMH and LH in obese rats. Nevertheless, the effect of exendin-4 on S6K activity in the VMH differed between obese and lean rats. Exendin-4 administration did not modify S6K activity in the

LH of lean and obese rats after four hours of re-feeding, whereas exendin-4 reduced S6K activity in the VMH of lean Zucker rats but not in their obese counterparts [83].

The prolonged activation of hypothalamic S6K inhibits insulin signalling and contributes to hepatic insulin resistance [150], suggesting that hypothalamic S6K activation would be involved in the pathogenesis of diet-induced hepatic insulin resistance. Our data indicate that S6K activity in the presence of exendin-4 could be decreased when this protein is maximally activated. This suggests that exendin-4 treatment in diabetic subjects could also improve hepatic insulin resistance.

7. Conclusions

We have reported here some of the many actions of GLP-1, such as, its role as an incretin hormone and controlling food intake. Accordingly, we have reviewed the importance of hypothalamic areas in the control of food intake, such as, for example, the ventromedial and lateral hypothalamus. In parallel, the function of AMPK and the mTOR/S6K pathway has been studied in those areas. Likewise, we have explored the coordinated response of hypothalamic AMPK and S6K to alterations in nutritional status and energy storage. Our results have revealed both the activation of AMPK and S6K in the VMH and LH in response to changes in glucose concentration or nutritional state, and that GLP-1/exendin-4 acts by counteracting the activation/inactivation of these kinases and contributing to the balance of proper AMPK and S6K activation. It therefore seems that GLP-1/exendin-4 might be acting in the VMH and LH, interacting with the AMPK/S6K signalling pathways, and modulating the activation status of AMPK and S6K in response to nutrient fluctuations. Likewise, GLP-1/exendin-4 would contribute to the normalization of the altered levels of these kinases in pathophysiological states such as obesity, for example.

Author details

Veronica Hurtado^{1,2,3}, Isabel Roncero^{1,2,3}, Enrique Blazquez^{1,2,3}, Elvira Alvarez^{1,2,3} and Carmen Sanz^{1,2,3,4}

1 Department of Biochemistry and Molecular Biology. Faculty of Medicine. University Complutense of Madrid, Spain

2 Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Spain

3 The Center for Biomedical Research in Diabetes and Associated Metabolic Disorders (CIBERDEM), Spain

4 Department of Cellular Biology. Faculty of Medicine. University Complutense of Madrid, Spain

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