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Glucagon-Like Peptide 1 (GLP-1) Can Reverse AMP-Activated Protein Kinase (AMPK) and S6 Kinase (P70S6K) Activities Induced by Fluctuations in Glucose Levels in Hypothalamic Areas Involved in Feeding Behaviour

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Abstract The anorexigenic peptide, glucagon-like peptide-1 (GLP-1), reduces glucose metabolism in the human hypothalamus and brain stem. The brain activity of metabolic sensors such as AMP-activated protein kinase (AMPK) responds to changes in glucose levels. The mammalian target of rapamycin (mTOR) and its downstream target, p70S6 kinase (p70S6K), integrate nutrient and hormonal signals. The hypothalamic mTOR/p70S6K pathway has been implicated in the control of feeding and the regulation of energy balances. Therefore, we investigated the coordinated effects of glucose and GLP-1 on the expression and activity of AMPK and p70S6K in the areas involved in the control of feeding. The effect of GLP-1 on the expression and activities of AMPK and p70S6K was studied in hypothalamic slice explants exposed to low- and high-glucose concentrations by quantitative real-time RT-PCR and by the quantification of active-phosphorylated protein levels by

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immunoblot. In vivo, the effects of exendin-4 on hypothalamic AMPK and p70S6K activation were analysed in male obese Zucker and lean controls 1 h after exendin-4 injection to rats fasted for 48 h or after re-feeding for 2–4 h. High-glucose levels decreased the expression of *Ampk* in the lateral hypothalamus and treatment with GLP-1 reversed this effect. GLP-1 treatment inhibited the activities of AMPK and p70S6K when the activation of these protein kinases was maximum in both the ventromedial and lateral hypothalamic areas. Furthermore, in vivo s.c. administration of exendin-4 modulated AMPK and p70S6K activities in those areas, in both fasted and re-fed obese Zucker and lean control rats.

Keywords AMPK · Control of feeding · Antidiabetogenic agents · Hypothalamus · mTOR/S6K · Zucker rats

Introduction

AMPK functions as a cellular energy sensor and is activated during energy depletion. AMPK activation occurs through an increase in the AMP/ATP ratio and triggers large number of downstream targets by stimulating ATP-generating, catabolic pathways and inhibiting anabolic pathways [1, 2]. AMPK is a heterotrimeric serine/threonine kinase consisting of a catalytic α-subunit encoded by 2 genes (α1 or α2), a β-subunit encoded by 2 genes (β1, β2) and a regulatory γ-subunit encoded by 3 genes (γ1, γ2, γ3) [3]. The use of AMPK-knockout mice deficient in the catalytic subunit has shown that while AMPKα1−/− mice have no metabolic alterations, AMPKα2−/− mice show insulin resistance and no apparent changes in body weight or food intake [4–6].

Hypothalamic AMPK has been suggested to play a role in the central regulation of food intake and energy balance. Thus, fasting increases and re-feeding decreases AMPK activity in several hypothalamic nuclei [7]. Control of food intake is also modulated by several neuropeptides that may regulate feeding behaviour in animals and humans by stimulating (orexigenic peptides) or inhibiting (anorexigenic peptides) food intake to maintain energy homeostasis and body weight. Hypothalamic AMPK is also regulated by several orexigenic and anorexigenic signals [7–9]. In this sense, hypothalamic AMPK activity is inhibited by anorexigenic peptides such as leptin and specifically affects AMPKα2 activity [7–9].

The mammalian target of rapamycin (mTOR) and its downstream target, p70S6K, integrate nutrient and hormonal signals and regulate protein synthesis, cell growth and proliferation in peripheral organs. The hypothalamic mTOR/p70S6K pathway has been implicated in the control of feeding and the regulation of energy balances [10]. mTOR is activated by glucose and amino acids, causing an inhibition of food intake. Thus, hypothalamic AMPK and mTOR respond to changes in glucose and other nutrients in the opposite sense, and their effects on the regulation of food intake may overlap. In peripheral organs, an overabundance of fuel alters the activity of metabolic sensors, leading to insulin resistance [11]. Deregulation of this signalling pathway in the hypothalamic centres involved in the control of feeding could be involved in the development of obesity and type 2 diabetes.

We have previously reported that GLP-1 is an anorexigenic peptide [12–16] that reduces cerebral glucose metabolism in human hypothalamus and brain stem [17]. Coexpression of the GLP-1 receptors, glucokinase and GLUT-2 in hypothalamic cells involved in feeding behaviour might play a role in glucose sensing [14, 18–20]. At least two kinds of glucose sensor neurons have been described in the brain. Glucose-excited neurons are mainly present in the ventromedial hypothalamus (VMH) and are excited by increased glucose levels in the extracellular space, with changes in their firing rates. In contrast, glucose-inhibited neurons, mainly present in the lateral hypothalamus (LH) area, are excited by decreases in glucose in the extracellular space. It has been previously suggested that AMPK plays a role in the glucose sensing effect of glucose-inhibited neurones [21]. Claret et al. have reported that specific removal of α2AMPK in hypothalamic pro-opiomelanocortin neurones or in hypothalamic agouti-related peptide neurones modifies the response to extracellular glucose changes, suggesting a role for AMPK as a common glucose sensor in these neurones [22]. In light of the above experimental evidence, we studied the possible interactions between the actions of GLP-1 and the hypothalamic AMPK and mTOR/p70S6K pathway. GLP-1 was seen to be 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 glucose levels after ingestion of a meal. Furthermore, the GLP-1 receptor agonist exendin-4 is one of the oral hypoglycaemic agents used in clinical practice [23] and is a long-acting agonist that also produces weight loss [24–26]. The increased prevalence of type 2 diabetes and obesity in recent years points to the importance of developing therapies able to integrate glycaemic control and food intake. Besides GLP-1, AMPK has been proposed as another possible target for hypoglycaemic drugs. Thus, we investigated the effects of GLP-1 on the expression and activity of AMPK in the VMH and LH areas. We have previously reported a distinctive pattern of glucokinase activities between those areas [27] as well as a distinctive response to glucose extracellular levels and a different modulation by orexigenic and anorexigenic peptides [8, 28]. In addition, the effect of subcutaneous administration of exendin-4 on the activity of hypothalamic AMPK and p70S6K under starvation and re-feeding

conditions was analysed in the VMH and LH areas of obese male and lean control Zucker rats.

Materials and Methods

Experimental Animals

All procedures involving animals were approved by the appropriate Institutional Review Committee and met the guidelines for the care of animals specified by the European Community. Same-aged male Wistar rats weighing 200–250 g, lean normal Fa/fa Zucker rats weighing 250–350 g and obese male Zucker (fa/fa) weighing 400–550 g rats (Charles River Laboratories) were fed ad libitum with a standard pellet diet and housed at a constant temperature (21°C) on a 12-h light–dark cycle, with lights on at 08:00 A.M.

Cell Cultures

Mouse neuroblastoma N2A cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Sigma-Aldrich) containing 4.5 g/l glucose. The GT1–7 cell line (generously provided by P Mellon, Department of Reproductive Medicine, School of Medicine, University of California, San Diego, CA, USA) is an immortalized GnRH-secreting cell line created from mouse hypothalamic neurosecretory cells [29] and was maintained in DMEM (Sigma-Aldrich) containing 4.5 g/l glucose. The media were supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% foetal bovine serum (FBS). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Procedure for Hypothalamic Slice Explant Cultures

Hypothalamic slices were obtained as described previously [27]. Briefly, male Wistar rats were killed by decapitation, and the brains were quickly removed and immersed in cold (4°C) MEM medium containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 20% heat-inactivated horse serum, 4 mM glutamine, 6.5 mg/ml glucose and 100 U/ml penicillin–streptomycin. The hypothalami were removed from the brain and sectioned at 300 μm thickness on a Mcllwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The slices were cultured at 37°C in an atmosphere containing 5% CO_2 for 5 h in MEM supplemented with 25 mM HEPES and Hank's salt-enriched with the above components in order to stabilize the cultures. Then, the slices were incubated in medium containing 2% FBS and 5.5 mM glucose for 16 h. Following this, the hypothalamic slices were fasted at 0.5 mM glucose for 2 h. The medium was removed and the slices were incubated for different times with either 0.5 or 10 mM glucose, in some cases adding 10 nM GLP-1 over the last 10 min. At the end of the incubations, special care was taken to identify and isolate (by micropunching) the VMH and LH areas according to the stereotaxic coordinates [30].

Zucker rats were fasted for 48 h. Some animals were re-fed for 2–4 h, and some of them were treated s.c. with Exendin-4 (250 ng/100 g body weight, Bachem) for 1 h. The hypothalami were removed from the brains and sectioned at 500 μm, and VMH and LH were isolated as described above and immediately lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.25 mM EDTA, pH 8.0, 10 mM NaF, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate) and a tablet of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), after which they were exposed to microwave irradiation for 5 s.

Real-Time Polymerase Chain Reaction (TaqMan® Assay)

Total RNA from rat hypothalamic slices were extracted with TRIZOL (Life Technologies, Barcelona, Spain). The mRNA levels of *Ampk-α2* and *18s RNA* were measured by real-time quantitative RT-PCR using Taqman probes (Applied Biosystems). The primers and probes (Online Resource 1) were designed with the Primer Express 2.0 software from Applied Biosystems.

AMPK and p70S6K Kinase Activity Assay and Detection by Western Blot

For the kinase activity assays, cells were cultured for 2 h in the presence of 0.5 mM of glucose. They were then incubated in medium containing either 0.5 or 10 mM of glucose for 2–4 h. Occasionally, cells were incubated in the presence or absence of different protein kinase or phosphatase inhibitors at the concentrations described in Table 1 for 30 min. In some cases, 10 nM GLP-1 was added during the last 10 min of the incubation. The cells were immediately lysed in RIPA buffer and exposed to microwave irradiation for 5 s. AMPK or p70S6K activation was detected by western blot using the antibodies described in Online Resource 2. Finally, the blots were scanned and quantified using Quantity One software (Biorad, GS800 Densitometer).

Statistical Analyses

All values are presented as means±SEM. Comparisons among groups were made using ANOVA. $P<0.05$ was considered statistically significant.

Table 1 Inhibitors of protein kinases and phosphatases

Inhibitors		Manufacturer	Concentration
PKA inhibitor	KT5720	Santa Cruz Biotechnology, Santa Cruz, CA, USA	200 nM
PKC inhibitor	Ro-318220	Bionova Científica, Madrid, Spain	10 mM
PI3K inhibitor	LY294002	Bionova Científica, Madrid, Spain	10 mM
MEK inhibitor	PD 98059	Calbiochem, Darmstadt, Germany	2 mM
mTOR inhibitor	Rapamicin	Santa Cruz Biotechnology, Santa Cruz, CA, USA	100 nM
Phosphatases inhibitors: PP2B and PP1	Okadaic acid sodium salt	Santa Cruz Biotechnology, Santa Cruz, CA, USA	10 mM 20 nM
CaMK inhibitor	KN62	Santa Cruz Biotechnology, Santa Cruz, CA, USA	3 mM
CaMKK inhibitor	STO-609	Sigma-Aldrich, Madrid, Spain	5 mM

Results

Glucose and GLP-1 Regulate AMPK Expression in Hypothalamic Slice Explants

The mRNA levels of *Ampk-α2* were measured with real-time RT-PCR in hypothalamic slice explants after treatment with different glucose concentrations (1, 2.5 or 10 mM) (Fig. 1a). High-glucose concentrations (10 mM) decreased, ≈58%, the expression of the mRNA coding AMPKα2 in the LH area. The presence of GLP-1 was able to recover the decreased expression of *AMPK* caused by high-glucose concentrations in LH area (Fig. 1b).

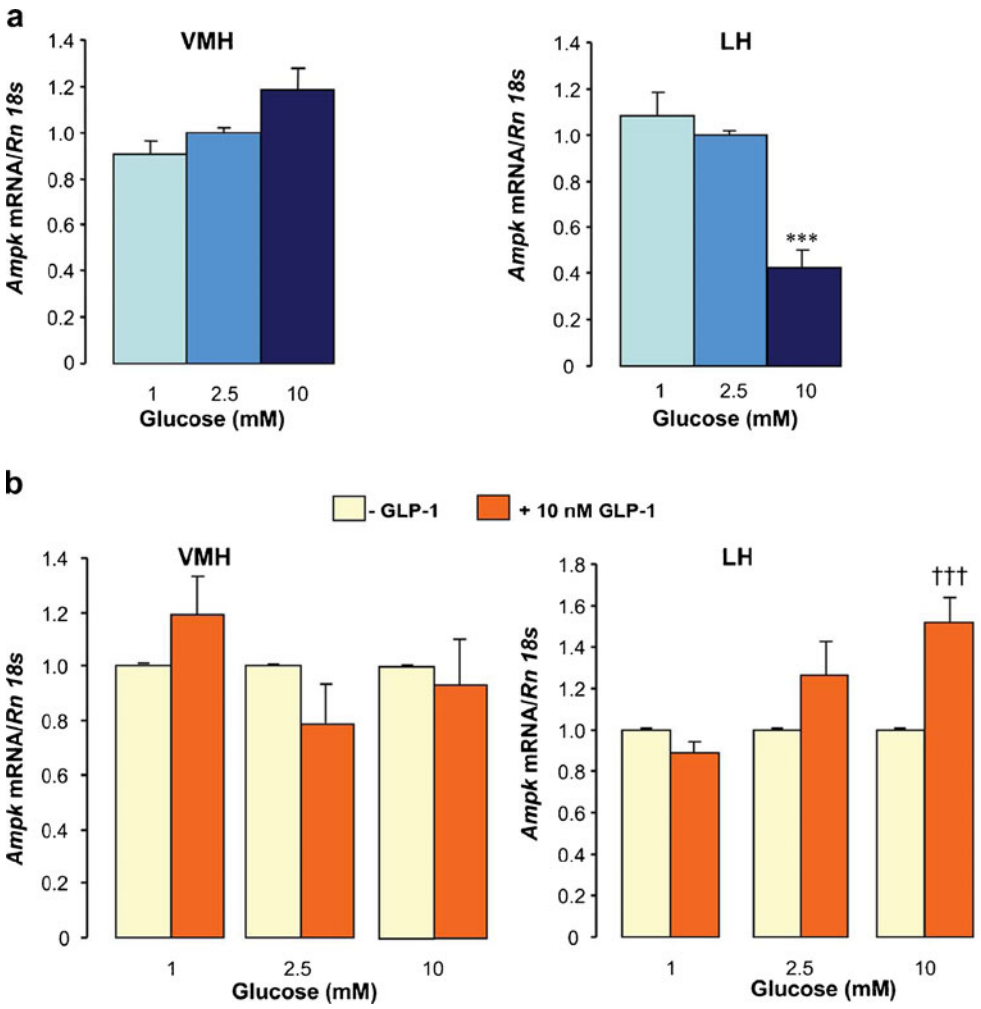

Fig. 1 Glucose and GLP-1 modulate *Ampk-α2* expression in the VMH and LH areas. Organotypic hypothalamic slices of 300 μm were glucose-starved for 2 h and then cultured for 3 h in a medium containing 1, 2.5 or 10 mmol/l glucose in the presence or absence of 10 nM GLP-1. The VMH and LH areas were dissected from slices. *Ampk-α2* mRNA was quantified by real-time RT-PCR analysis. **a** *Bars* represent *Ampk-α2* mRNA levels normalized by *RNA 18s* and referred to the value obtained under the 2.5-mM glucose condition, considered as 1. **b** *Bars* represent *Ampk-α2* mRNA levels normalized by *RNA 18s* and referred to the value obtained in absence of GLP-1 that was considered as 1. Data are expressed as means±SEM; $n=4$–5 independent experiments performed in duplicate. ***$P<0.001$ 10 mM glucose vs 2.5 mM glucose, $^{\dagger\dagger\dagger}P<0.001$, absence of GLP-1 vs the presence of 10 nM GLP-1

Glucose and GLP-1 Modulate AMPK and p70S6K Activities in Hypothalamic Slice Explants

Using hypothalamic slices, we tested whether GLP-1 modulated AMPK and p70S6K activities in the VMH and LH after exposure to low (0.5 mM)- and high (10 mM)-glucose concentrations in the presence or absence of 10 nM GLP-1. The activation of AMPK was checked using an anti-phospho-AMPKα (Thr172). Low-glucose concentrations increased the activity of AMPK in both hypothalamic nuclei by ≈2-fold (Fig. 2a), while GLP-1 treatment reversed the low-glucose effect (Fig. 2a).

The activation of p70S6K was detected using anti-phospho-p70S6K (Thr389). High-glucose levels increased p70S6K activity in VMH and LH by ≈3–10-fold (Fig. 2b), and the presence of GLP-1 reversed that activation.

Glucose and GLP-1 Modulate AMPK and p70S6K Activities in Hypothalamic GT1–7 and Neuroblastoma N2A Cell Lines

We used GT1–7 immortalized hypothalamic neurons and a neuroblastoma cell line to confirm the effects of low- or high-glucose concentrations on AMPK and p70S6K activities. We found that AMPK phosphorylation was dependent on the experimental conditions used to prepare the cell lysates. Initially, we detected considerable variability from 1 day to the other of the experiments. The degree of AMPK phosphorylation at different glucose concentrations was significantly different when the cell lysates were subjected or not to microwave irradiation for 5 s (Fig. 3). Our data suggested that the process employed to make up the cell extracts was a definitive step in obtaining reproducible results. At least in the case of AMPK, the level of the phosphorylated forms increased after cell lysis, even in the presence of Laemmli sample buffer (Fig. 3 and Online Resource 3). Exposure to microwave irradiation for 5–10 s immediately after the addition of cell lysis buffer was seen to be the most efficient method for maintaining the level of protein phosphorylation.

Using this cell lysis procedure in GT1–7 cells, we observed that the AMPK activities increased at low-glucose concentration (Fig. 4a) while the activation of p70S6K was seen at high-glucose concentrations (Fig. 4b). The presence of GLP-1 decreased the AMPK activity previously stimulated by low-glucose concentrations to a significant extent (Fig. 4a). This anorexigenic peptide also markedly attenuated the activation of p70S6K observed at high-glucose concentrations (Fig. 4b).

Low-glucose concentrations produced a rapid activation of AMPK in the N2A cells that persisted over time (Fig. 4c), and this activation was significantly inhibited by GLP-1 (Fig. 4c). However, the activation of p70S6K required incubations of more than 2 h with high glucose (Fig. 4d), and the presence of GLP-1 also significantly decreased p70S6K activation (Fig. 4d).

Mechanisms of GLP-1 Signalling in the Activities of Metabolic Sensors

In a first attempt to elucidate the signalling pathways downstream from the GLP-1 receptor that mediate the regulation of hypothalamic metabolic sensors, we used several specific inhibitors of different protein kinases and phosphatases that could be involved in the signalling mechanism. The state of activation of AMPK remained unchanged in the presence of most of the inhibitors (Fig. 5a). However, the inhibitory effect of GLP-1 on AMPK phosphorylation was reversed in the presence of the protein phosphatase inhibitors PP1 and PP2B and inhibitors of the protein kinases PKA, PKC and PI3K (Fig. 5b).

Exendin-4 Regulates Hypothalamic AMPK and p70S6K Activities In Vivo in Obese and Lean Control Zucker Rats

The restriction of food for 48 h increased AMPK activity in the VMH and LH areas in both lean control and obese Zucker rats (Fig. 6). However, in both areas, AMPK activity was ≈40–47% lower in the obese rats than in lean Zucker animals. Two hours of re-feeding after 48 h of fasting reduced by ≈5-fold AMPK activity in both areas in all groups (Fig. 6 and Table 2). The effect of fasting on AMPK activity in Zucker lean controls was reversed by exendin-4 administration for 1 h, whereas the effect of exendin-4 in obese Zucker rats did not modify AMPK activity significantly. The reduction in AMPK activity observed in the VMH and LH areas, after re-feeding for 2 h, was reversed by exendin-4 treatment in both the obese and lean control rats (Fig. 6 and Table 2). Four hours of re-feeding reversed the AMPK activity of the VMH area up to fasted levels in both lean and obese rats (Fig. 6 and Table 2). Nevertheless, the AMPK activity of the LH area remained low in the lean rats (Fig. 6 and Table 2).

We also analysed p70S6K activity under the same conditions. Two hours of re-feeding, after 48 h of food deprivation, reduced p70S6K activity by ≈2–3-fold in both areas in all groups, and 4 h later, p70S6K activity increased by ≈2–4-fold as compared to 2 h (Fig. 7 and Table 3). The effect of exendin-4 administration in the fasted rats did not change p70S6K activity significantly (Fig. 7 and Table 3). After of 2 h re-feeding, exendin-4 treatment increased p70S6K activity in the VMH and LH in all groups. Nevertheless, the effect of exendin-4 on p70S6K activity in the VMH differed in the obese and lean rats. While exendin-4 administration did not modify p70S6K activity in lean rats to any significant extent, it dramatically increased p70S6K activity in obese rats. However, no significant effect was observed for exendin-4

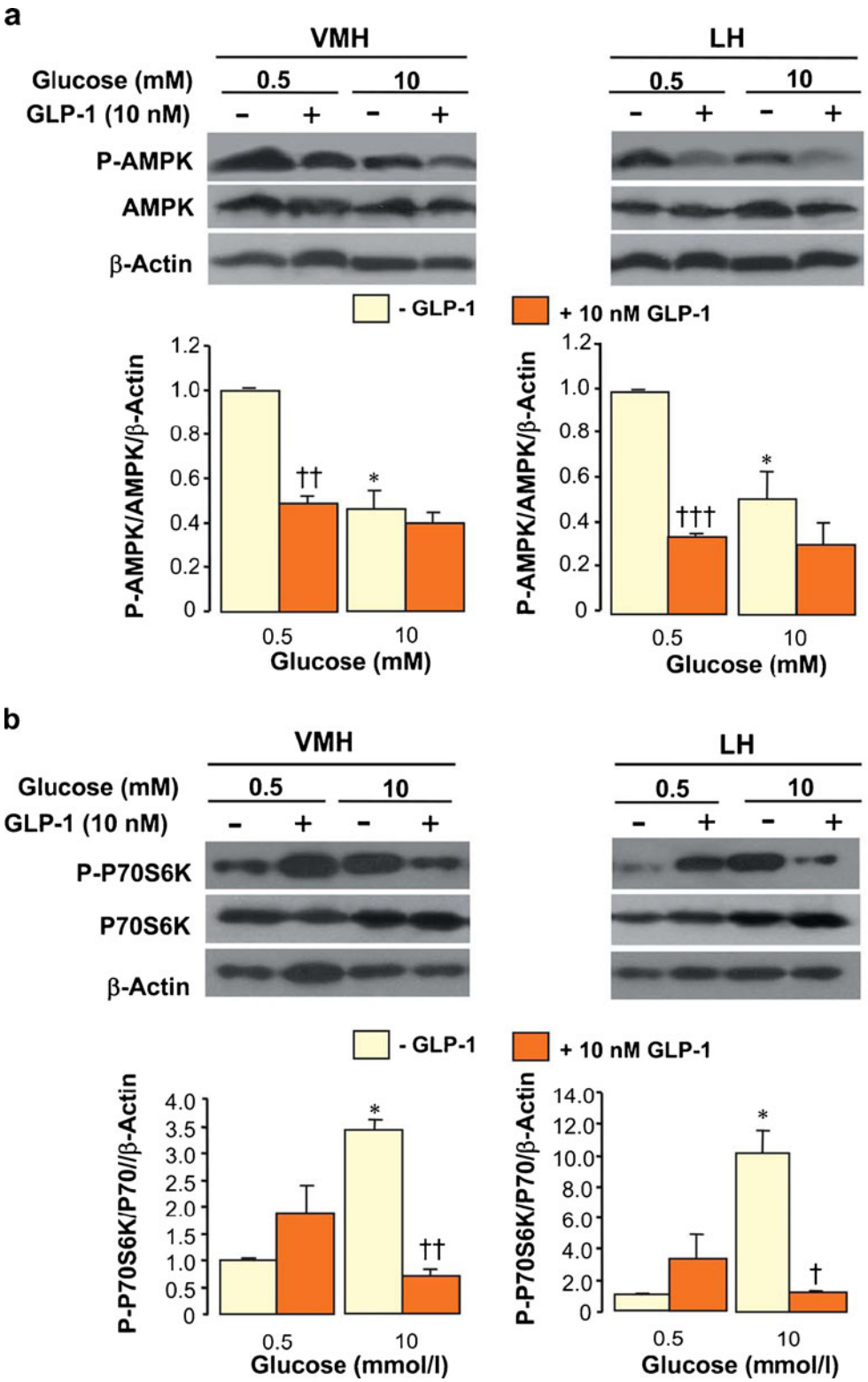

Fig. 2 Glucose and GLP-1 regulated AMPK and p70S6K activities in the VMH and LH areas. Hypothalamic slice explants were glucose-starved for 2 h and then cultured for 2 h in DMEM containing 2% FBS, 4 mM glutamine, 100 U/ml penicillin–streptomycin and either 0.5 or 10 mM glucose. In some cases, 10 nM GLP-1 was added during the last 10 min of culture. The VMH and LH areas were isolated by micropunching, lysed in RIPA buffer and exposed for 5 s to microwave irradiation and then processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK) (**a**) and phospho-p70S6K (Thr-389) (P-P70S6K) and total p70S6K (P70S6K) (**b**). The blots were reprobed for β-actin. Densitometric values normalized by β-actin and by non-phosphorylated forms and referred to the value of 0.5 mM glucose without GLP-1 in VMH, taken as 1. Results are means±SEM; $n=3$. *$P<0.05$ 10 mM glucose vs 0.5 mM glucose and $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$, absence of GLP-1 vs the presence of 10 nM GLP-1

administration after 4 h of re-feeding on p70S6K activity in the LH of lean and obese rats, while the exendin-4 reduced p70S6K activity in the VMH of lean but not of obese Zucker rats.

Discussion

In recent years, AMPK has been proposed as a cellular energy sensor that is able to assemble many regulatory

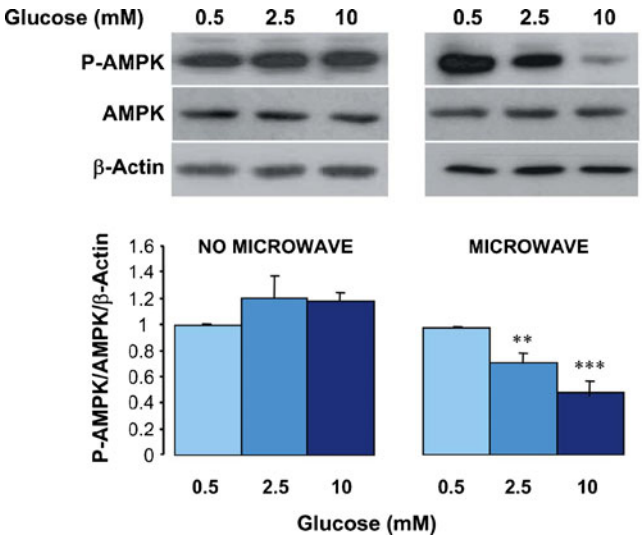

Fig. 3 Importance of the microwave irradiation in the stability of the phosphorylated AMPK forms in the cell-lysate. N2A cells were cultured in DMEM/F-12 containing 10% FBS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) and 0.5 mM glucose for 2 h. Then, the medium was removed and the cells were incubated for 2 h in a medium containing 0.5, 2.5 or 10 mM glucose. N2A cells were immediately lysed in RIPA buffer and exposed or not to microwave irradiation for 5 s, and then Laemmli buffer was added and processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK). The blots were reprobed for β-Actin. Densitometric values were normalized by β-actin and by non-phosphorylated forms. The value obtained in the cells treated with 0.5 mM glucose was taken as 1. The results are means±SEM; n=4–5. $^{**}P<0.01$, $^{***}P<0.001$ as compared with 0.5 mM glucose

signals and nutritional environmental changes, and it is also involved in maintaining whole-body energy balance [31]. The regulation of AMPK activity located in the hypothalamic areas involved in the control of feeding behaviour has also been described as a mechanism for the detection of nutritional variations, including glucose levels [32]. The results of several studies have indicated that hypothalamic AMPK activity is regulated by glucose levels and by changes in nutrients and hormones responding to the nutritional status. In general, AMPK is activated by fasting and is inhibited by re-feeding [7], but in the hypothalamus hypoglycaemia induces the activation of hypothalamic AMPK, specifically in the VMH and PVN but not in the LH (32). mTOR is one of the downstream targets of AMPK in which elevated glucose concentrations activate the mTOR/p70S6K pathway and cause an inhibition of food intake. In the present study, we confirm the notion that the effects of glucose on AMPK and p70S6K are region-specific in hypothalamic areas that have opposite effects over the control of feeding behaviour. These effects were found both in vitro, using hypothalamic slices, and in vivo, in lean and obese Zucker rats, and it was also observed that GLP-1 was able to modulate AMPK and p70S6K activities, depending on the state of activation.

Rat hypothalamic slices have previously been reported to preserve some tissue architecture and functional connections, maintaining a selective c-Fos expression in response to different glucose concentrations [27] similar to the functional activity found in vivo [33]. Sanz et al. reported a different response to glucose in the VMH and LH [27, 28]. Using the same experimental approach, here we show that high-glucose levels decrease the expression of *Ampk-α2*, specifically in the LH. In previous work [34], changes in *Ampk* expression have been reported. Thus, 3 days of insulin-induced hypoglycaemia produced an increase in the gene expression of *Ampk* throughout the hypothalamus, and Seo et al. reported an increase in *Ampk* mRNA in the hypothalamus of fasted rats and that the intracerebroventricular administration of GLP-1 attenuated such an effect [35]. Our results confirm the idea that GLP-1 treatment is able to modulate *Ampk-α2* expression and indicate a distinctive response in the LH as compared to the VMH. This result can be explained in terms of the different roles of these two areas in the control of food intake, previously defined as centres of hunger and satiety respectively. It is generally accepted that pharmacological activation of AMPK in the liver and muscle may elicit a decrease in blood glucose and lipid levels that could be useful in the treatment of type 2 diabetes [36].

Our results suggest that increased glucose levels, which could be similar to those found in uncontrolled diabetic patients, may modulate not only AMPK activity but also the expression of *Ampk*, possibly in a cell-specific way in different brain areas. It has been reported that in normoglycaemic rats, the extracellular glucose concentration in the brain was ~2.5 mM and increased to ~4.5 mM at blood glucose levels of ~15 mM [37]. Previous data using neuroblastoma cells indicated that ATP concentration increased markedly in a range of 1–5 mM glucose and the concentration of ATP above of 5 mM glucose was maintained stable [38]. In our study, we have used 0.5 mM as low-glucose concentration and 10 mM as high-glucose concentration. Apparently, 10 mM glucose could be considered too high in brain, although the blood glucose levels in obese animals can be higher than 20 mM [15].

Previously, it has been reported that anorexigenic peptides decrease AMPKα2 activity [7, 39]. Other authors have also reported that insulin and leptin increase the phosphorylation of p70S6K and that treatment with rapamycin, a mTOR inhibitor, blocks the effect of leptin [10].

In our study, GLP-1 also modulated AMPK and p70S6K activities in the VMH and LH. An interesting finding was that the effect of this peptide was always dependent on the activation status of AMPK and p70S6K, requiring maximum activation of these sensors to exert it. Thus, at low-glucose concentrations, AMPK activity was stimulated and p70S6K activity was maintained with minimal activation,

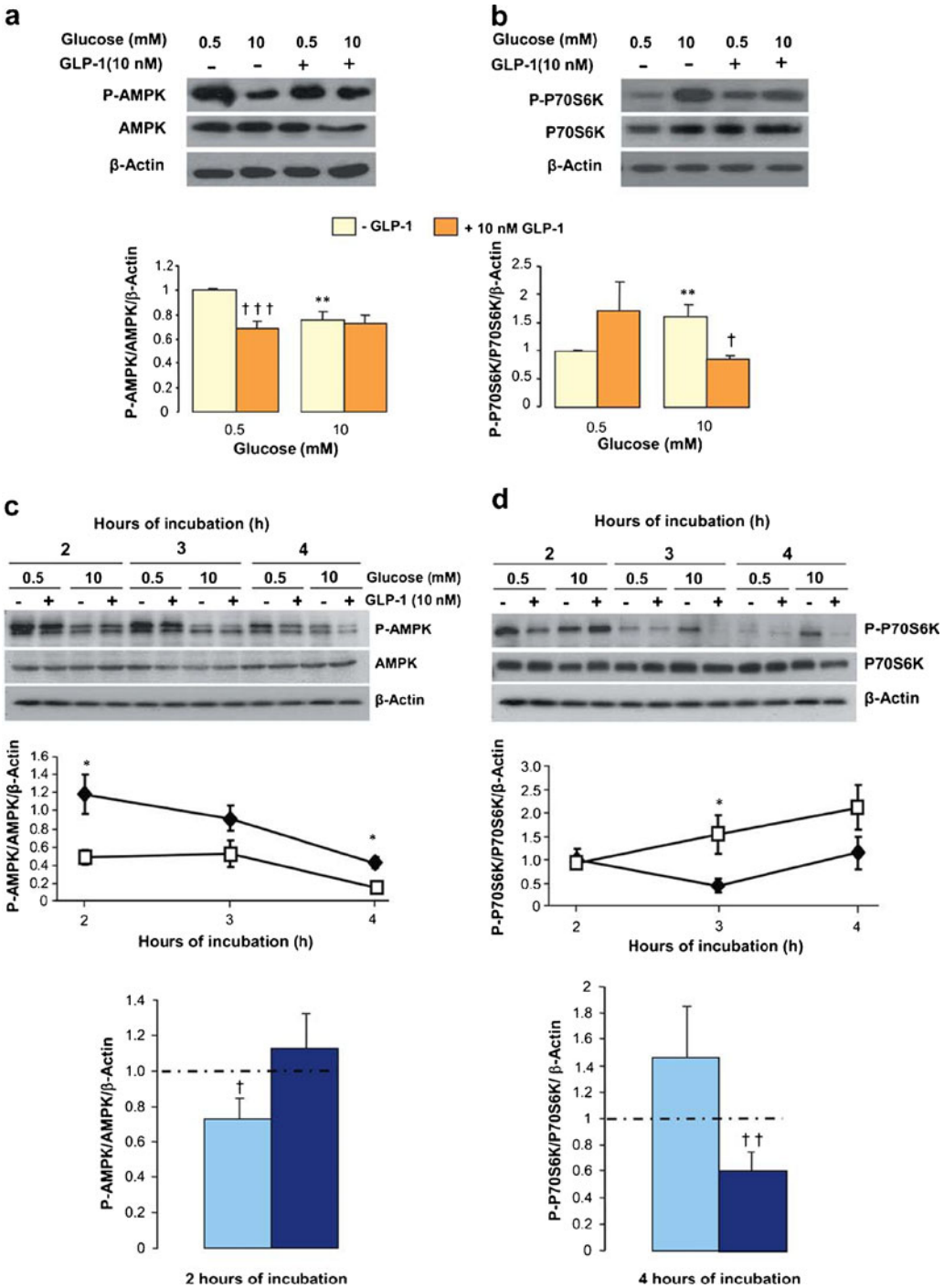

Fig. 4 Glucose and GLP-1 modulate AMPK and p70S6K activation in the hypothalamic GT1–7 and neuroblastoma N2A cell lines. GT1–7 and N2A cells were cultured in DMEM or DMEM/F-12, respectively, containing 10% FBS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) and 0.5 mM glucose for 2 h. Then, the medium was removed and the cells were incubated for 2, 3 or 4 h in a medium containing 0.5 or 10 mM glucose. Finally, cells were treated or not with 10 nM GLP-1 for 10 min. GT1–7 cells were immediately lysed in RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK) (**a**) and phospho-p70S6K (Thr-389) (P-P70S6K) and total p70S6K (P70S6K) (**b**). N2A cells were immediately lysed in RIPA buffer and exposed to microwave irradiation for 5 s and then processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK) (**c**) and phospho-p70S6K (Thr-389) (P-P70S6K) and total p70S6K (P70S6K) (**d**). The blots were reprobed for β-Actin. Densitometric values, normalized by β-actin and non-phosphorylated forms, were referred to a value of 0.5 mM glucose 2 h without GLP-1, taken as 1. The *line graphs* represent the effect of glucose on AMPK and p70S6K activation at different incubation times. The results are means± SEM; n=4–5. *P<0.05; 10 mM glucose vs 0.5 mM glucose. The *bar graphs* represent the effect of GLP-1 on AMPK and p70S6K activation. *Horizontal line* indicates the absence of GLP-1. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 absence of GLP-1 vs presence of 10 nM GLP-1

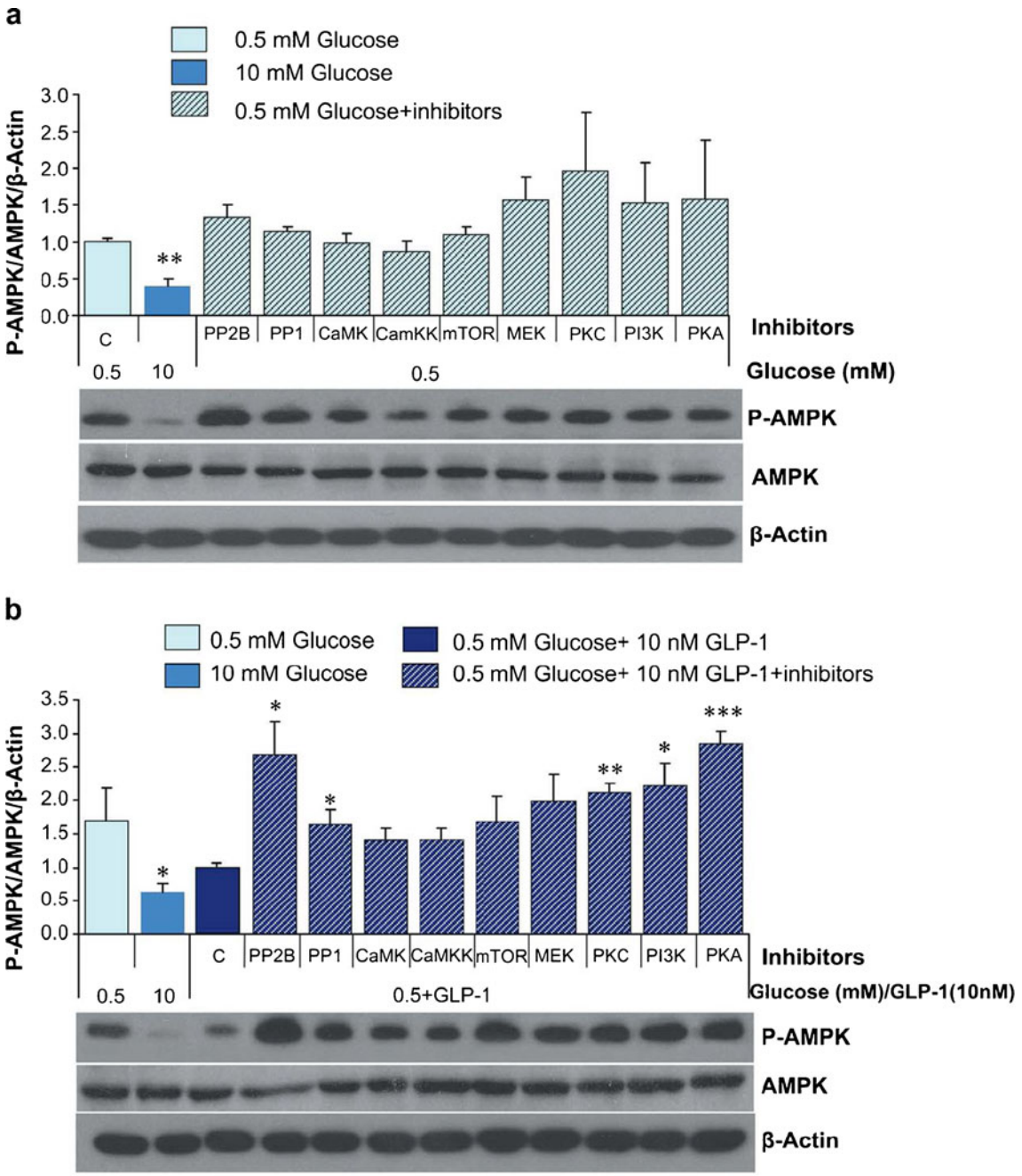

Fig. 5 Impact of several protein kinases and phosphatases inhibitors on the GLP-1-induced inhibition of AMPK activity at low-glucose concentrations. N2A cells were incubated in the presence of 0.5 mM glucose 2 h, treated or not with several inhibitors for 30 min (Table 1), in the absence (**a**) or presence (**b**) of 10 nM GLP-1 over the last 10 min. Cell lysates were exposed to microwave irradiation for 5 s and then processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK) (**a**, **b**). Densitometric values were normalized by β-actin and by non-phosphorylated forms. **a** The value of 0.5 mM glucose without inhibitors was taken as 1 (**c**). The results are means±SEM; n=3–4. **P<0.01, 10 mM glucose compared with 0.5 mM glucose. **b** The value obtained in the cells treated with 0.5 mM glucose and 10 nM GLP-1 without inhibitors (**c**) was taken as 1. The results are means±SEM; n=3–4. *P<0.05, **P<0.01, ***P< 0.001 vs without inhibitors

while GLP-1 treatment reversed the effect of glucose on AMPK and did not modify p70S6K activity in the VMH and LH. High levels of glucose led to the activation of p70S6K 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. The metabolic sensors in these cells respond to glucose as described above and GLP-1

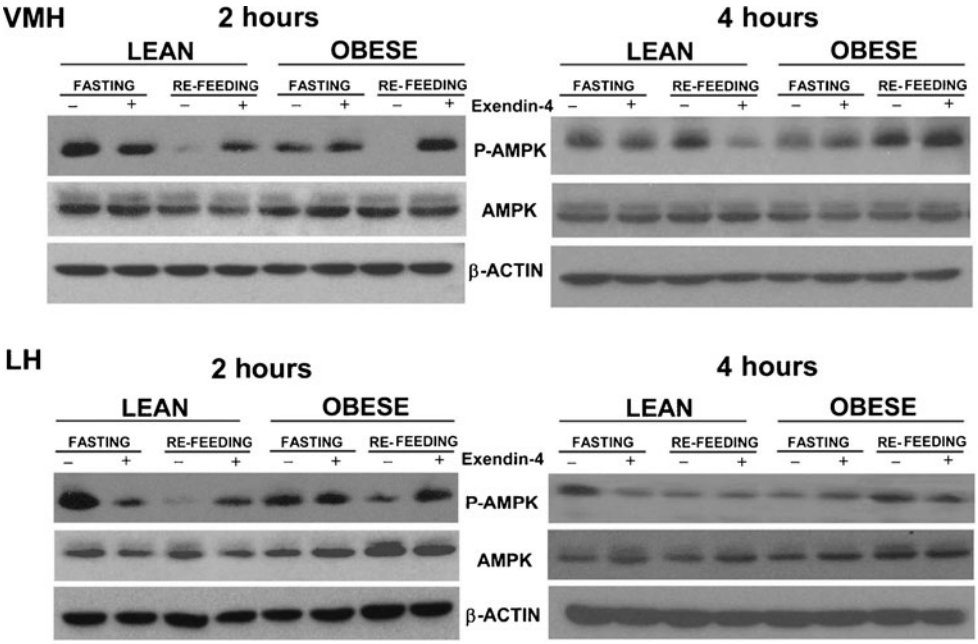

Fig. 6 Exendin-4 modulated hypothalamic AMPK activity in obese and lean control Zucker rats. Animals were food-deprived for 48 h, after which they were re-fed for 2 or 4 h and some of them were treated s.c. with exendin-4 (250 ng/100 g body weight or vehicle for 1 h). The VMH and LH hypothalamic areas were isolated by micropunching. Tissues were lysed in RIPA buffer, exposed to microwave irradiation for 5 s and then processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK). The blots were reprobed for β-actin

treatment reversed de glucose effects. For this analysis, the cells grown in high (25 mM)-glucose medium were serum-starved in medium containing 0.5 mM glucose for 2 h in order to initiate the stimulation in more physiological conditions. The cells showed no apparent signs of glucotoxicity in accordance with previous studies using N2A maintained in medium containing 25 mM glucose as control in studies of toxicity [40].

Here we report that the level of AMPK protein phosphorylation increased even after cell lysis in the presence of Laemmli sample buffer. In order to arrest any changes in the phosphorylation of the cell homogenates after cell lysis, the extracts were always immediately exposed to microwave irradiation. This method proved to be the most efficient way to maintain the level of protein phosphorylation, in accordance with a previously reported method of tissue preparation [41].

The signalling pathways targeted by GLP-1 that are involved in the modulation of hypothalamic metabolic sensors remain largely unknown. As a first approach to elucidating some of these protein kinases or phosphatases that might be involved in the actions of GLP-1,

Table 2 Hypothalamic AMPK activity in obese and lean control Zucker rats in response to different nutritional status and exendin-4 treatment

		Zucker rats			
		Lean		Obese	
Hypothalamic area	Nutritional status	Untreated	Exendin-4	Untreated	Exendin-4
VMH	Fasting	1.00	0.53±0.10[g]	1.00 (0.53±0.1[c])	1.51±0.25
	Re-feeding 2 h	0.23±0.063[b]	2.17±0.15[g]	0.20±0.08[b]	2.63±0.18[h]
	Re-feeding 4 h	1.18±0.08[e]	0.29±0.01[h]	1.34±0.29[e]	1.19±0.07
LH	Fasting	1.00	0.35±0.08[h]	1.00 (0.59±0.09[c])	0.95±0.11
	Re-feeding 2 h	0.18±0.11[b]	6.42±1.68[f]	0.12±0.04[b]	2.49±0.16[h]
	Re-feeding 4 h	0.39±0.08[a]	0.73±0.18	1.29±0.24[d]	0.91±0.10

Animals were food-deprived for 48 h, after which they were re-fed for 2 or 4 h and some of them were treated s.c. with exendin-4 (250 ng/100 g body weight or vehicle for 1 h). Data expressed as the densitometric values of phospho-AMPK (Thr-172) normalized by total AMPK and β-actin (Fig. 6). Results are means±SEM. The data represent the response to nutritional status (untreated) relative to the value obtained in lean and obese fasted animals, both VMH and LH, taken as 1; n=3–9

[a] $P<0.05$; [b] $P<0.001$ (re-feeding vs fasting); [c] $P<0.001$ (values in parentheses fasting obese rats vs fasting lean rats); [d] $P<0.01$; [e] $P<0.001$ (re-feeding 4 h vs re-feeding 2 h); [f] $P<0.05$; [g] $P<0.01$; [h] $P<0.001$ (exendin-4 treated vs untreated)

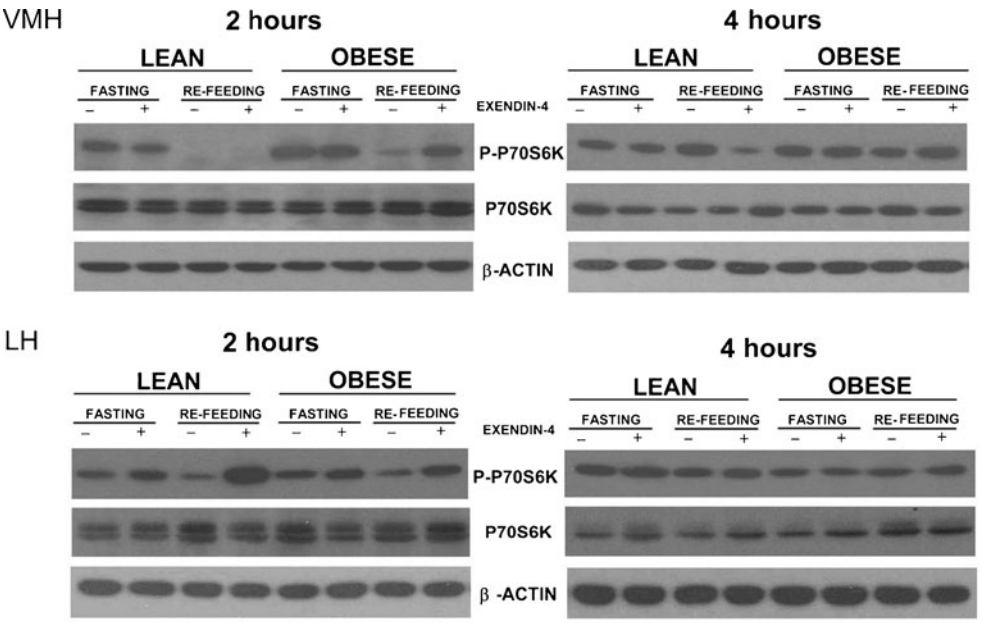

Fig. 7 Exendin-4 modulated hypothalamic p70S6K activity in obese and lean control Zucker rats. Animals were food-deprived for 48 h, after which they were re-fed for 2 or 4 h and some of them were treated s.c. with exendin-4 (250 ng/100 g body weight or vehicle for 1 h). The VMH and LH hypothalamic areas were isolated by micropunching. Tissues were lysed in RIPA buffer, exposed to microwave irradiation for 5 s and then processed for western blot analysis. The levels of phospho-p70S6K (Thr-389) (P-P70S6K) and total p70S6K (P70S6K) were analysed by western blot. The blots were reprobed for β-actin

we used specific inhibitors in the N2A cell line. The results obtained suggest that the signalling pathway initiated at the GLP-1 receptor may be mediated through the activation of PKA, PKC and PI3K and that GLP-1 would enhance the effect of the PP2 inhibitor to a significant extent. These results are in accordance with recent findings reported by Hayes et al. [42], relating increased PKA and mitogen-activated protein kinase activity to the suppression of food intake by GLP-1 in the nucleus of the tractus solitaries, or those indicating that the anorexic actions of insulin or leptin can be blocked by the inhibition of PI3K and that kinase mediates mTOR and p70S6K activation [43, 44]. Several reports have also described that the regulation of AMPK activity depends on the activity of protein phosphatases [45, 46].

A further step was to use an in vivo model involving Zucker rats to analyse the effect of the GLP-1 agonist on hypothalamic AMPK and p70S6K activities. The obese Zucker (fa/fa) rat offers a well-established animal model of insulin resistance and genetic obesity and, in comparison with lean Zucker rat, exhibits hyperinsulinemia and

Table 3 Hypothalamic p70S6K activity in obese and lean control Zucker rats in response to different nutritional status and exendin-4 treatment

		Zucker rats			
		Lean		Obese	
Hypothalamic area	Nutritional status	Untreated	Exendin-4	Untreated	Exendin-4
VMH	Fasting	1.00	1.41±0.41	1.00 (1.22±0.23)	0.83±0.09
	Re-feeding 2 h	0.47±0.10[b]	1.61±0.60	0.37±0.17[a]	5.60±0.53[f]
	Re-feeding 4 h	1.70±0.36[d]	0.28±0.06[e]	1.54±0.40[c]	1.11±0.17
LH	Fasting	1.00	0.95±0.15	1.00 (0.78±0.22)	1.34±0.33
	Re-feeding 2 h	0.28±0.08[b]	3.46±0.51[e]	0.54±0.12[a]	2.50±0.35[e]
	Re-feeding 4 h	0.74±0.08[c]	0.77±0.09	1.22±0.20[c]	1.01±0.14

Animals were food-deprived for 48 h, after which they were re-fed for 2 or 4 h and some of them were treated s.c. with exendin-4 (250 ng/100 g body weight or vehicle for 1 h). Data are expressed as the densitometric values of phospho-p70S6K (Thr-389) normalized by total p70S6K and β-actin (Fig. 7). Results are means±SEM. The data represent the response to nutritional status (untreated) relative to the value obtained in lean and obese fasted animals, both VMH and LH, taken as 1; n=3–7. Values in parentheses fasting obese rats vs fasting lean rats

[a] $P<0.01$; [b] $P<0.001$ (re-feeding vs fasting); (values in parentheses fasting obese rats vs fasting lean rats); [c] $P<0.05$; [d] $P<0.01$ (re-feeding 4 h vs re-feeding 2 h); [e] $P<0.01$; [f] $P<0.001$ (exendin-4 treated vs untreated)

hyperlipidemia. We have previously described that peripheral long-term s.c. administration of exendin-4 decreased food intake and induced weight loss in both obese and lean control Zucker rats [15]. Our results showed that AMPK activity in the VMH and LH from lean and obese rats was increased during starvation and that it was inhibited at 2 h after re-feeding as reported previously [7, 8]. Notably, the level of AMPK activation was lower in the obese than in lean Zucker rats in both areas. This result could be due to alterations in the levels of nutrients and hormones in obese rats. An unexpected result was obtained upon analysing the effect of fasting and re-feeding on p70S6K activity. This decreased significantly in the VMH and LH of animals that were re-fed for 2 h as compared to fasted animals. The activation of p70S6K was only observed after 4 h of re-feeding. The analysis of p70S6K activity in response to high-glucose levels in N2A cells also showed that the response to an increase in glucose levels required at least a period of 3–4 h. Cota et al. [10] have previously reported a decrease in phospho-p70S6K in fasted rats as compared with rats re-fed for 3 h. It is possible that in our experiments, maximum activation of p70S6K was not detected and thus could account for the lack of significant differences between the p70S6K activities observed in fasted animals and those re-fed for 4 h. Our data indicate that at least with our experimental design, the lowest level of p70S6K activity in the VMH and LH was present in rats that were re-fed for 2 h.

Here we show that the effect of fasting on hypothalamic AMPK activity from lean Zucker rats is reversed by s.c. exendin-4 administration for 1 h, whereas the effect of exendin-4 on fasted obese Zucker rats does not significantly modify AMPK activity. This could compensate the decrease in AMPK activity observed in the VMH and LH in obese Zucker as compared to lean control rats. Our results also indicate that exendin-4 could activate AMPK when AMPK activity is strongly inhibited, as in animals re-fed for 2 h. Accordingly, GLP-1 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. The complexities of the regulation of hypothalamic AMPK activity under different feeding conditions have been described previously for some hormones. Thus, ghrelin or cannabinoids have ad libitum effects [47], whereas leptin [7] and adiponectin [48] only have an effect after variable times of fasting or re-feeding. The cocaine- and amphetamine-regulated transcript (CART) has been reported to have anorexic effect after intracerebroventricular administration [49], while CART injected directly into the paravetricular or arcuate nucleus of fasted rats increases food intake [50].

Previous work has indicated that anorexic peptides enhance the mTOR/p70S6K pathway, leading to inhibition of food intake. Cota et al. reported that insulin and leptin increase the phosphorylation of p70S6K [10]. Here we report that exendin-4 modulates p70S6K activity, and it is indeed remarkable that the effect of exendin-4 depends on the activation status of p70S6K, as occurred with AMPK. Thus, exendin-4 stimulates p70S6K activity in animals re-fed for 2 h, these animals showing the lowest activation of p70S6K, while—in contrast—exendin-4 decreases p70S6K activity in the VMH of lean rats re-fed for 4 h to a significant extent.

Ono et al. [51] have suggested that hypothalamic p70S6K activation would be involved in the pathogenesis of diet-induced hepatic insulin resistance. The prolonged activation of hypothalamic p70S6K produces the inhibition of insulin signalling and contributes to hepatic insulin resistance. Our data indicate that in the presence of exendin-4, p70S6K activity could be decreased when this protein is maximally activated. This suggests that exendin-4 treatment in diabetic subjects also could improve hepatic insulin resistance.

Finally, the results reported here indicate that in the VMH and LH areas, GLP-1 modulates the activation status of AMPK and p70S6K in response to variations in glucose or in pathological states such as obesity and insulin resistance. We also present experimental evidence of some of the kinases or phosphatases that may mediate these GLP-1 actions. Also, the data obtained suggest a potential role for GLP-1 or exendin-4 as preservers of the hypothalamic AMPK and p70S6K activities in some pathological states.

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Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Hardie DG, Carling D, Carlson M (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 67:821–855
2. Rutter GA, Da Silva XG, Leclerc I (2003) Roles of 5′-AMP-activated protein kinase (AMPK) in mammalian glucose homoeostasis. Biochem J 375(Pt 1):1–16

3. Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 8 (10):774–785
4. Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, Vaulont S, Richter EA, Wojtaszewski JF (2004) Knockout of the alpha2 but not alpha1 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. J Biol Chem 279(2):1070–1079
5. Viollet B, Andreelli F, Jorgensen SB, Perrin C, Geloen A, Flamez D, Mu J, Lenzner C, Baud O, Bennoun M, Gomas E, Nicolas G, Wojtaszewski JF, Kahn A, Carling D, Schuit FC, Birnbaum MJ, Richter EA, Burcelin R, Vaulont S (2003) The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. J Clin Invest 111(1):91–98
6. Viollet B, Athea Y, Mounier R, Guigas B, Zarrinpashneh E, Horman S, Lantier L, Hebrard S, Devin-Leclerc J, Beauloye C, Foretz M, Andreelli F, Ventura-Clapier R, Bertrand L (2009) AMPK: lessons from transgenic and knockout animals. Front Biosci 14:19–44
7. Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferre P, Birnbaum MJ, Stuck BJ, Kahn BB (2004) AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428(6982):569–574
8. Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, Yun JY, Namgoong IS, Ha J, Park IS, Lee IK, Viollet B, Youn JH, Lee HK, Lee KU (2004) Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nat Med 10(7):727–733
9. Lim CT, Kola B, Korbonits M (2010) AMPK as a mediator of hormonal signalling. J Mol Endocrinol 44(2):87–97
10. Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, Seeley RJ (2006) Hypothalamic mTOR signaling regulates food intake. Science 312(5775):927–930
11. Vodenik B, Rovira J, Campistol JM (2009) Mammalian target of rapamycin and diabetes: what does the current evidence tell us? Transplant Proc 41(6 Suppl):S31–S38
12. Alvarez E, Roncero I, Chowen JA, Thorens B, Blazquez E (1996) Expression of the glucagon-like peptide-1 receptor gene in rat brain. J Neurochem 66(3):920–927
13. Blazquez E, Alvarez E, Navarro M, Roncero I, Rodriguez-Fonseca F, Chowen JA, Zueco JA (1998) Glucagon-like peptide-1 (7–36) amide as a novel neuropeptide. Mol Neurobiol 18(2):157–173
14. Navarro M, Rodriquez de Fonseca F, Alvarez E, Chowen JA, Zueco JA, Gomez R, Eng J, Blazquez E (1996) Colocalization of glucagon-like peptide-1 (GLP-1) receptors, glucose transporter GLUT-2, and glucokinase mRNAs in rat hypothalamic cells: evidence for a role of GLP-1 receptor agonists as an inhibitory signal for food and water intake. J Neurochem 67 (5):1982–1991
15. Rodriguez de Fonseca F, Navarro M, Alvarez E, Roncero I, Chowen JA, Maestre O, Gomez R, Munoz RM, Eng J, Blazquez E (2000) Peripheral versus central effects of glucagon-like peptide-1 receptor agonists on satiety and body weight loss in Zucker obese rats. Metabolism 49(6):709–717
16. Turton MD, O'Shea D, Gunn I, Beak SA, Edwards CM, Meeran K, Choi SJ, Taylor GM, Heath MM, Lambert PD, Wilding JP, Smith DM, Ghatei MA, Herbert J, Bloom SR (1996) A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379(6560):69–72
17. Alvarez E, Martinez MD, Roncero I, Chowen JA, Garcia-Cuartero B, Gispert JD, Sanz C, Vazquez P, Maldonado A, de Caceres J, Desco M, Pozo MA, Blazquez E (2005) The expression of GLP-1 receptor mRNA and protein allows the effect of GLP-1 on glucose metabolism in the human hypothalamus and brainstem. J Neurochem 92(4):798–806
18. Alvarez E, Roncero I, Chowen JA, Vazquez P, Blazquez E (2002) Evidence that glucokinase regulatory protein is expressed and interacts with glucokinase in rat brain. J Neurochem 80(1):45–53
19. Roncero I, Alvarez E, Chowen JA, Sanz C, Rabano A, Vazquez P, Blazquez E (2004) Expression of glucose transporter isoform GLUT-2 and glucokinase genes in human brain. J Neurochem 88(5):1203–1210
20. Roncero I, Alvarez E, Vazquez P, Blazquez E (2000) Functional glucokinase isoforms are expressed in rat brain. J Neurochem 74 (5):1848–1857
21. Mountjoy PD, Bailey SJ, Rutter GA (2007) Inhibition by glucose or leptin of hypothalamic neurons expressing neuropeptide Y requires changes in AMP-activated protein kinase activity. Diabetologia 50(1):168–177
22. Claret M, Smith MA, Batterham RL, Selman C, Choudhury AI, Fryer LG, Clements M, Al-Qassab H, Heffron H, Xu AW, Speakman JR, Barsh GS, Viollet B, Vaulont S, Ashford ML, Carling D, Withers DJ (2007) AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. J Clin Invest 117 (8):2325–2336
23. Niswender K (2010) Diabetes and obesity: therapeutic targeting and risk reduction—a complex interplay. Diabetes Obes Metab 12 (4):267–287
24. Blonde L, Klein EJ, Han J, Zhang B, Mac SM, Poon TH, Taylor KL, Trautmann ME, Kim DD, Kendall DM (2006) Interim analysis of the effects of exenatide treatment on A1C, weight and cardiovascular risk factors over 82 weeks in 314 overweight patients with type 2 diabetes. Diabetes Obes Metab 8(4):436–447
25. Buse JB, Rosenstock J, Sesti G, Schmidt WE, Montanya E, Brett JH, Zychma M, Blonde L (2009) Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6). Lancet 374(9683):39–47
26. Montanya E, Sesti G (2009) A review of efficacy and safety data regarding the use of liraglutide, a once-daily human glucagon-like peptide 1 analogue, in the treatment of type 2 diabetes mellitus. Clin Ther 31(11):2472–2488
27. Sanz C, Roncero I, Vazquez P, Navas MA, Blazquez E (2007) Effects of glucose and insulin on glucokinase activity in rat hypothalamus. J Endocrinol 193(2):259–267
28. Sanz C, Vazquez P, Navas MA, Alvarez E, Blazquez E (2008) Leptin but not neuropeptide Y up-regulated glucagon-like peptide 1 receptor expression in GT1–7 cells and rat hypothalamic slices. Metabolism 57(1):40–48
29. Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI (1990) Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5(1):1–10
30. Paxinos G, Watson C (2004) The rat brain in stereotaxic coordinates. Elsevier, New York
31. Hardie DG, Hawley SA, Scott JW (2006) AMP-activated protein kinase—development of the energy sensor concept. J Physiol 574 (Pt 1):7–15
32. Mountjoy PD, Rutter GA (2007) Glucose sensing by hypothalamic neurones and pancreatic islet cells: AMPle evidence for common mechanisms? Exp Physiol 92(2):311–319
33. Solomon A, De Fanti BA, Martinez JA (2006) Peripheral ghrelin participates in the glucostatic signaling mediated by the ventromedial and lateral hypothalamus neurons. Peptides 27(7):1607–1615
34. McCrimmon RJ, Shaw M, Fan X, Cheng H, Ding Y, Vella MC, Zhou L, McNay EC, Sherwin RS (2008) Key role for AMP-activated protein kinase in the ventromedial hypothalamus in regulating counterregulatory hormone responses to acute hypoglycemia. Diabetes 57(2):444–450
35. Seo S, Ju S, Chung H, Lee D, Park S (2008) Acute effects of glucagon-like peptide-1 on hypothalamic neuropeptide and AMP activated kinase expression in fasted rats. Endocr J 55(5):867–874

36. Long YC, Zierath JR (2006) AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 116(7):1776–1783
37. Silver IA, Erecinska M (1994) Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. J Neurosci 14 (8):5068–5076
38. Lee K, Li B, Xi X, Suh Y, Martin RJ (2005) Role of neuronal energy status in the regulation of adenosine 5′-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. Endocrinology 146(1):3–10
39. Andersson U, Filipsson K, Abbott CR, Woods A, Smith K, Bloom SR, Carling D, Small CJ (2004) AMP-activated protein kinase plays a role in the control of food intake. J Biol Chem 279 (13):12005–12008
40. Manzoni C, Colombo L, Bigini P, Diana V, Cagnotto A, Messa M, Lupi M, Bonetto V, Pignataro M, Airoldi C, Sironi E, Williams A, Salmona M (2011) The molecular assembly of amyloid abeta controls its neurotoxicity and binding to cellular proteins. PLoS One 6(9):e24909
41. Scharf MT, Mackiewicz M, Naidoo N, O'Callaghan JP, Pack AI (2008) AMP-activated protein kinase phosphorylation in brain is dependent on method of killing and tissue preparation. J Neurochem 105(3):833–841
42. Hayes MR, Leichner TM, Zhao S, Lee GS, Chowansky A, Zimmer D, De Jonghe BC, Kanoski SE, Grill HJ, Bence KK (2011) Intracellular signals mediating the food intake-suppressive effects of hindbrain glucagon-like peptide-1 receptor activation. Cell Metab 13(3):320–330
43. Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, Schwartz MW (2001) Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature 413(6858):794–795
44. Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG Jr, Seeley RJ, Schwartz MW (2003) Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes 52 (2):227–231
45. Woods A, Vertommen D, Neumann D, Turk R, Bayliss J, Schlattner U, Wallimann T, Carling D, Rider MH (2003) Identification of phosphorylation sites in AMP-activated protein kinase (AMPK) for upstream AMPK kinases and study of their roles by site-directed mutagenesis. J Biol Chem 278(31):28434–28442
46. Garcia-Haro L, Garcia-Gimeno MA, Neumann D, Beullens M, Bollen M, Sanz P (2010) The PP1-R6 protein phosphatase holoenzyme is involved in the glucose-induced dephosphorylation and inactivation of AMP-activated protein kinase, a key regulator of insulin secretion, in MIN6 beta cells. Faseb J 24(12):5080–5091
47. McCrimmon RJ, Fan X, Cheng H, McNay E, Chan O, Shaw M, Ding Y, Zhu W, Sherwin RS (2006) Activation of AMP-activated protein kinase within the ventromedial hypothalamus amplifies counterregulatory hormone responses in rats with defective counterregulation. Diabetes 55(6):1755–1760
48. Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, Kozono H, Takamoto I, Okamoto S, Shiuchi T, Suzuki R, Satoh H, Tsuchida A, Moroi M, Sugi K, Noda T, Ebinuma H, Ueta Y, Kondo T, Araki E, Ezaki O, Nagai R, Tobe K, Terauchi Y, Ueki K, Minokoshi Y, Kadowaki T (2007) Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab 6(1):55–68
49. Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393(6680):72–76
50. Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, Zollner AN, Morgan DG, Morgan I, Ghatei MA, Small CJ, Bloom SR (2001) Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. Endocrinology 142(8):3457–3463
51. Ono H, Pocai A, Wang Y, Sakoda H, Asano T, Backer JM, Schwartz GJ, Rossetti L (2008) Activation of hypothalamic S6 kinase mediates diet-induced hepatic insulin resistance in rats. J Clin Invest 118(8):2959–2968