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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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# 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 down-stream 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 immunoblot. *In vivo*, the effects of exendin-4 on hypothalamic AMPK and p70S6K activation were analyzed 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.

**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 (Hardie et al. 1998; Rutter et al. 2003).

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) (Hardie 2007).

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 (Jorgensen et al. 2004; Viollet et al. 2003; Viollet et al. 2009).

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 (Minokoshi et al. 2004). 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 (Kim et al. 2004; Lim et al. 2010; Minokoshi et al. 2004). In this sense, hypothalamic AMPK activity is inhibited by anorexigenic peptides such as leptin and specifically affects AMPKα2 activity (Kim et al. 2004; Lim et al. 2010; Minokoshi et al. 2004).

The mammalian target of rapamycin (mTOR) and its down-stream 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 (Cota et al. 2006). 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 (Vodenik et

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al. 2009). 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 (Alvarez et al. 1996; Blazquez et al. 1998; Navarro et al. 1996; Rodriguez de Fonseca et al. 2000; Turton et al. 1996) that reduces cerebral glucose metabolism in human hypothalamus and brain stem (Alvarez et al. 2005). Coexpression of the GLP-1 receptors, glucokinase and GLUT-2 in hypothalamic cells involved in feeding behaviour might play a role in glucose sensing (Alvarez et al. 2002; Navarro et al. 1996; Roncero et al. 2004; Roncero et al. 2000). 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 (Mountjoy et al. 2007). Claret et al. have reported that specific removal of α2AMPK ~~knocked out~~ in hypothalamic pro-opiomelanocortin neurones or in hypothalamic agouti-related peptide neurones modifies the response~~did not respond~~ to extracellular glucose changes, suggesting a role for AMPK as a common glucose-sensor in these neurones (Claret et al. 2007). 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 decreases 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 (Niswender 2010) and is a long-acting agonist that also produces weight loss (Blonde et al. 2006; Buse et al. 2009; Montanya and Sesti 2009). 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

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activities between thoese areas (Sanz et al. 2007) as well as a distinctive response to glucose extracellular levels and a different modulation by orexigenic and anorexigenic peptides (Kim et al. 2004; Sanz et al. 2008).

In addition, the effect of subcutaneous administration of exendin-4 on the activity of hypothalamic AMPK and p70S6K under starvation and re-feeding conditions were analyzed 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 (21ºC) on a 12-h light-dark cycle, with lights on at 08:00 A.M.

### Cell cultures

Mouse neuroblastoma N2A cells were grown in 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 a immortalized GnRH-secreting cell line created ~~transformed~~ from mouse hypothalamic neurosecretory cells (Mellon et al. 1990), was maintained in DMEM (Sigma-Aldrich) containing 4.5 g/l glucose. The media were supplemented with 2 mM-glutamine, penicillin (100U/mL), streptomycin (100 mg/ml), and 10% 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 (Sanz et al. 2007). Briefly, male Wistar rats were killed by decapitation and the brains were quickly removed and immersed in cold (4ºC) MEM medium containing 25 mM 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

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sectioned at 300 µm thickness on a Mcllwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The slices were cultured at 37º 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 mM 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 (Paxinos and Watson 2004).

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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, VMH and LH were isolated as described above and immediately lysed in 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 hours in the presence of 0.5 mM of glucose. They were then incubated in medium containing either 0.5 mM 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.

## 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).

### 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 one 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 two hours 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 analyzed 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 administration after 4h 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 signals and nutritional environmental changes, and it is also involved in maintaining whole-body energy balance (Hardie et al. 2006). 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 (Mountjoy and Rutter 2007). 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 (Minokoshi et al. 2004), 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 (Sanz et al. 2007) similar to the functional activity found *in vivo* (Solomon et al. 2006). Sanz et al. reported a different response to glucose in the VMH and LH (Sanz et al. 2007; Sanz et al. 2008). 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 (McCrimmon et al. 2008), 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 (Seo et al. 2008). 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

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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 (Long and Zierath 2006).

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 normoglycemic 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 (Silver and Erecinska 1994). 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 (Lee et al. 2005). 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 (Rodriguez de Fonseca et al. 2000).

Previously, it has been reported that anorexigenic peptides decrease AMPKα2 activity (Andersson et al. 2004; Minokoshi et al. 2004). 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 (Cota et al. 2006).

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, 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 treatment reversed de glucose effects. For this analysis the cells grown in high (25 mM) glucose medium

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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 sings of glucotoxicity in accordance with previous studies using N2A maintained in medium containing 25 mM glucose as control in studies of toxicity (Manzoni et al. 2011).

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 (Scharf et al. 2008).

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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 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 (Hayes et al. 2011), relating increased PKA and MAPK 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 (Niswender et al. 2001; Niswender et al. 2003). Several reports have also described that the regulation of AMPK activity depends of the activity of protein phosphatases (Woods et al. 2003; Garcia-Haro et al. 2010).

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A further step was to use an *in vivo* model involving Zucker rats to analyze 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 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 (Rodriguez de Fonseca et al. 2000). 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 2h

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after re-feeding as reported previously (Kim et al. 2004; Minokoshi et al. 2004). 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 analyzing 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 (Cota et al. 2006) 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 (McCrimmon et al. 2006), whereas leptin (Minokoshi et al. 2004), adiponectin (Kubota et al. 2007) 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 (Kristensen et al. 1998), while CART injected directly into the paravetricular or arcuate nucleus of fasted rats increases food intake (Abbott et al. 2001).

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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 (Cota et al. 2006). 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 2h, 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.

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Ono et al (Ono et al. 2008) 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.

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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 not conflict of interest.

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**LEGENDS TO FIGURES**

**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

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**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

**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, 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

**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 hours 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 minutes (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.

**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.

**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 analyzed by western blot. The blots were reprobed for β-Actin.

**Table 1.** Inhibitors of protein kinases and phosphatases

| Inhibitors | | Manufacturer | Concentration |
|---|---|---|---|
| PKA inhibitor | KT5720 | Santa Cruz Biotechnology, California, 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, Alemania | 2 mM |
| mTOR inhibitor | Rapamicin | Santa Cruz Biotechnology, California, USA | 100 nM |
| Phosphatases inhibitors: PP2B and PP1 | Okadaic Acid Sodium Salt | Santa Cruz Biotechnology, California, USA | 10 mM<br>20 nM |
| CaMK inhibitor | KN62 | Santa Cruz Biotechnology, California, USA | 3 mM |
| CaMKK inhibitor | STO-609 | Sigma Aldrich, Madrid, Spain | 5 mM |

Table 2
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**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** | |
| **Hypotalamic area** | **Nutritional status** | **Untreated** | **Exendin-4** | **Untreated** | **Exendin-4** |
| **VMH** | Fasting | 1.00 | $0.53 \pm 0.10^{\S\S}$ | 1.00 ($0.53 \pm 0.1^{\dagger\dagger\dagger}$) | $1.51 \pm 0.25$ |
| | Re-feeding 2 h | $0.23 \pm 0.063^{***}$ | $2.17 \pm 0.15^{\S\S}$ | $0.20 \pm 0.08^{***}$ | $2.63 \pm 0.18^{\S\S\S}$ |
| | Re-feeding 4 h | $1.18 \pm 0.08^{\ddagger\ddagger\ddagger}$ | $0.29 \pm 0.01^{\S\S\S}$ | $1.34 \pm 0.29^{\ddagger\ddagger\ddagger}$ | $1.19 \pm 0.07$ |
| **LH** | Fasting | 1.00 | $0.35 \pm 0.08^{\S\S\S}$ | 1.00 ($0.59 \pm 0.09^{\dagger\dagger\dagger}$) | $0.95 \pm 0.11$ |
| | Re-feeding 2 h | $0.18 \pm 0.11^{***}$ | $6.42 \pm 1.68^{\S}$ | $0.12 \pm 0.04^{***}$ | $2.49 \pm 0.16^{\S\S\S}$ |
| | Re-feeding 4 h | $0.39 \pm 0.08^{*}$ | $0.73 \pm 0.18$ | $1.29 \pm 0.24^{\ddagger\ddagger}$ | $0.91 \pm 0.10$ |

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. $*P<0.05$, $***P<0.001$ re-feeding vs fasting; $^{\dagger\dagger\dagger}P<0.001$ values in parentheses fasting obese rats vs fasting lean rats; $^{\ddagger\ddagger}P<0.01$, $^{\ddagger\ddagger\ddagger}P<0.001$ re-feeding 4 h vs re-feeding 2 h; $^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$ exendin-4 treated vs untreated.

Table 3
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**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 | |
| **Hypotalamic 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$^{***}$ | 1.61 ± 0.60 | 0.37 ± 0.17$^{**}$ | 5.60 ± 0.53$^{§§§}$ |
| | Re-feeding 4 h | 1.70 ± 0.36$^{‡‡}$ | 0.28 ± 0.06$^{§§}$ | 1.54 ± 0.40$^{‡}$ | 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$^{***}$ | 3.46 ± 0.51$^{§§}$ | 0.54 ± 0.12$^{**}$ | 2.50 ± 0.35$^{§§}$ |
| | Re-feeding 4 h | 0.74 ± 0.08$^{‡}$ | 0.77 ± 0.09 | 1.22 ± 0.20$^{‡}$ | 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. $^{**}P<0.01$, $^{***}P<0.001$ re-feeding vs fasting; values in parentheses fasting obese rats vs fasting lean rats; $^{‡}P<0.05$, $^{‡‡}P<0.01$ re-feeding 4 h vs re-feeding 2 h; $^{§§}P<0.01$, $^{§§§}P<0.001$ exendin-4 treated vs untreated.

Figure 1
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Fig 1

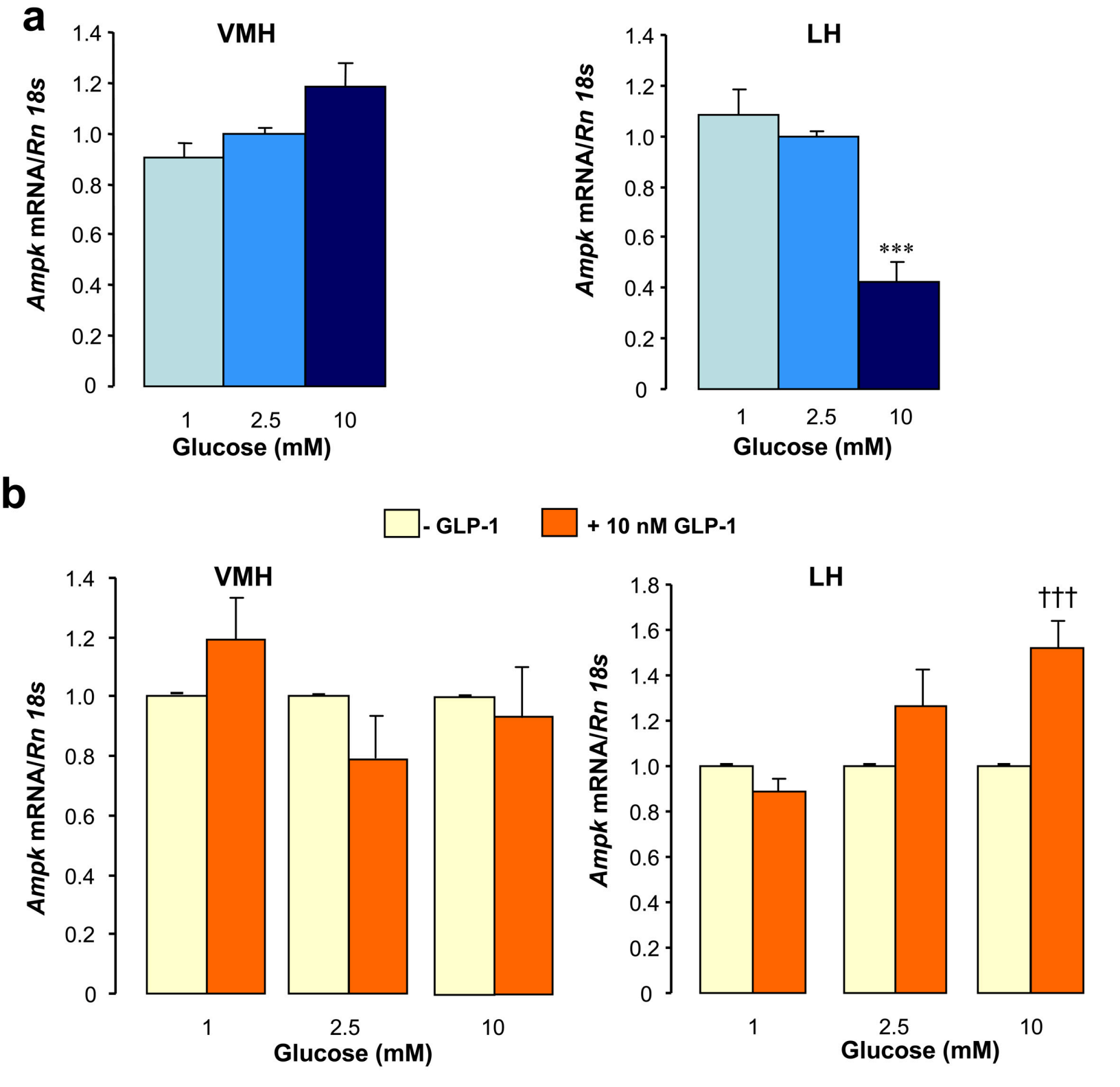

Figure 2

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Fig. 2

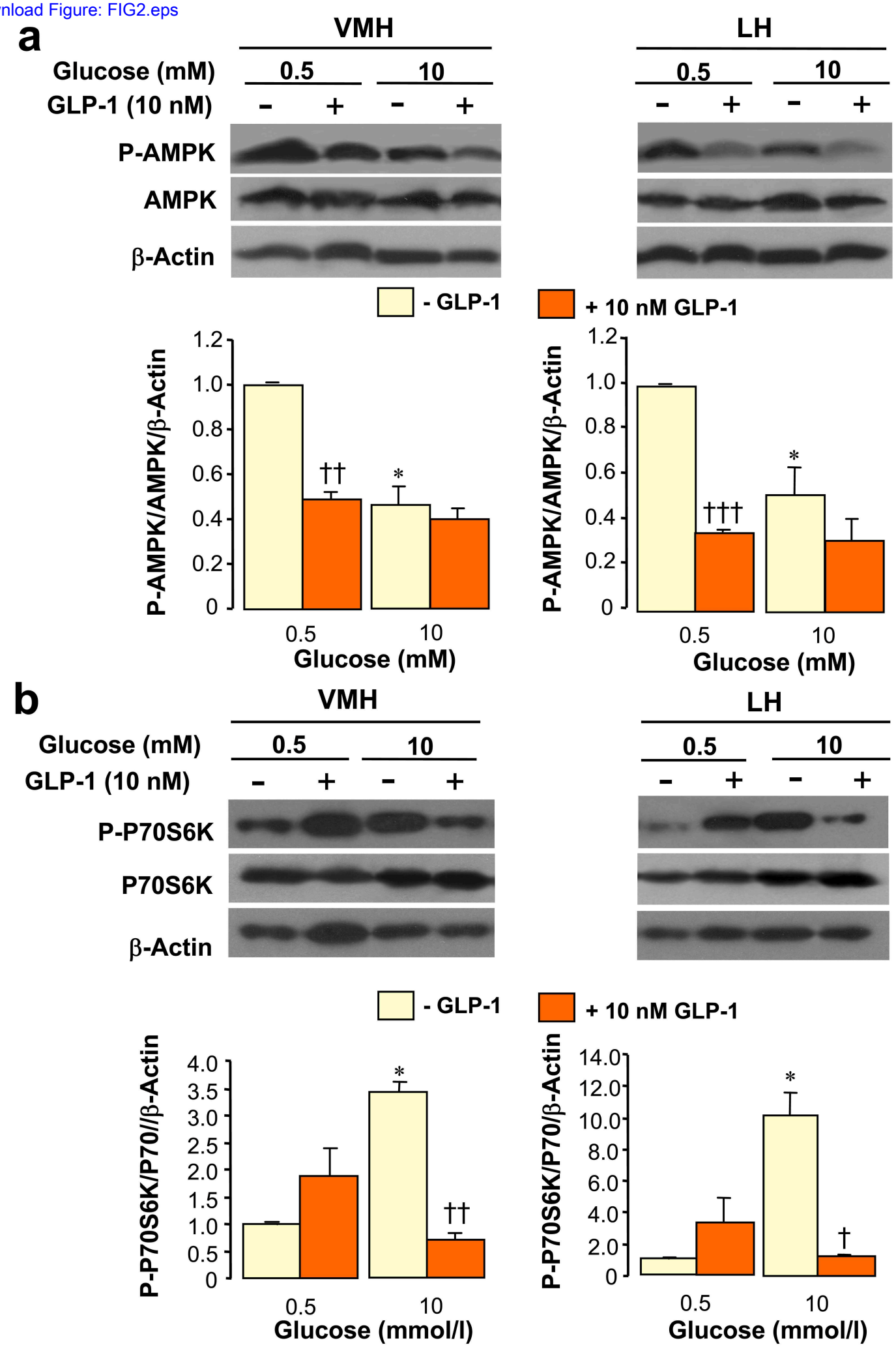

Figure 3
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# Fig 3

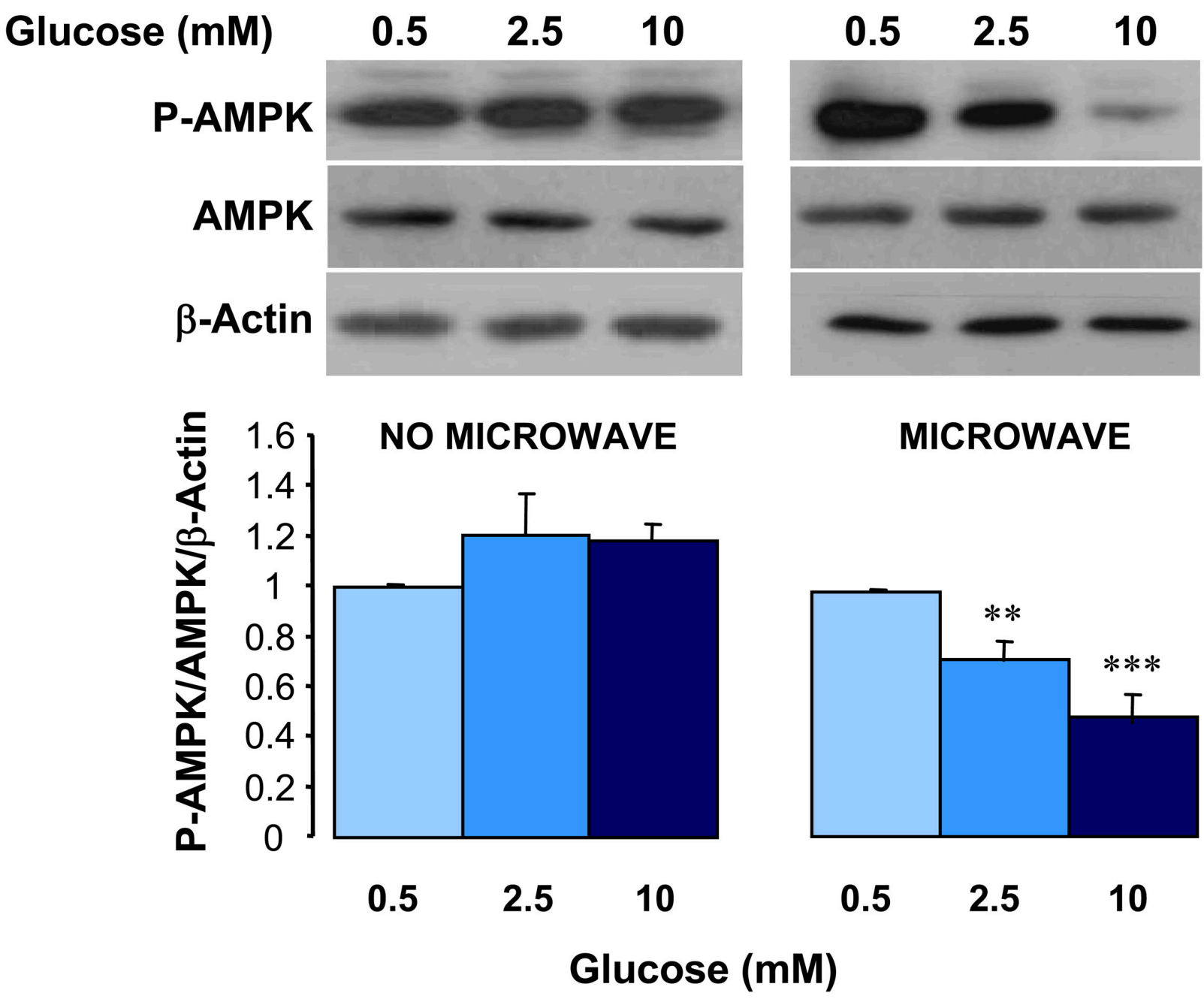

Figure 4

Fig 4

Click here to download Figure: FIG4.eps

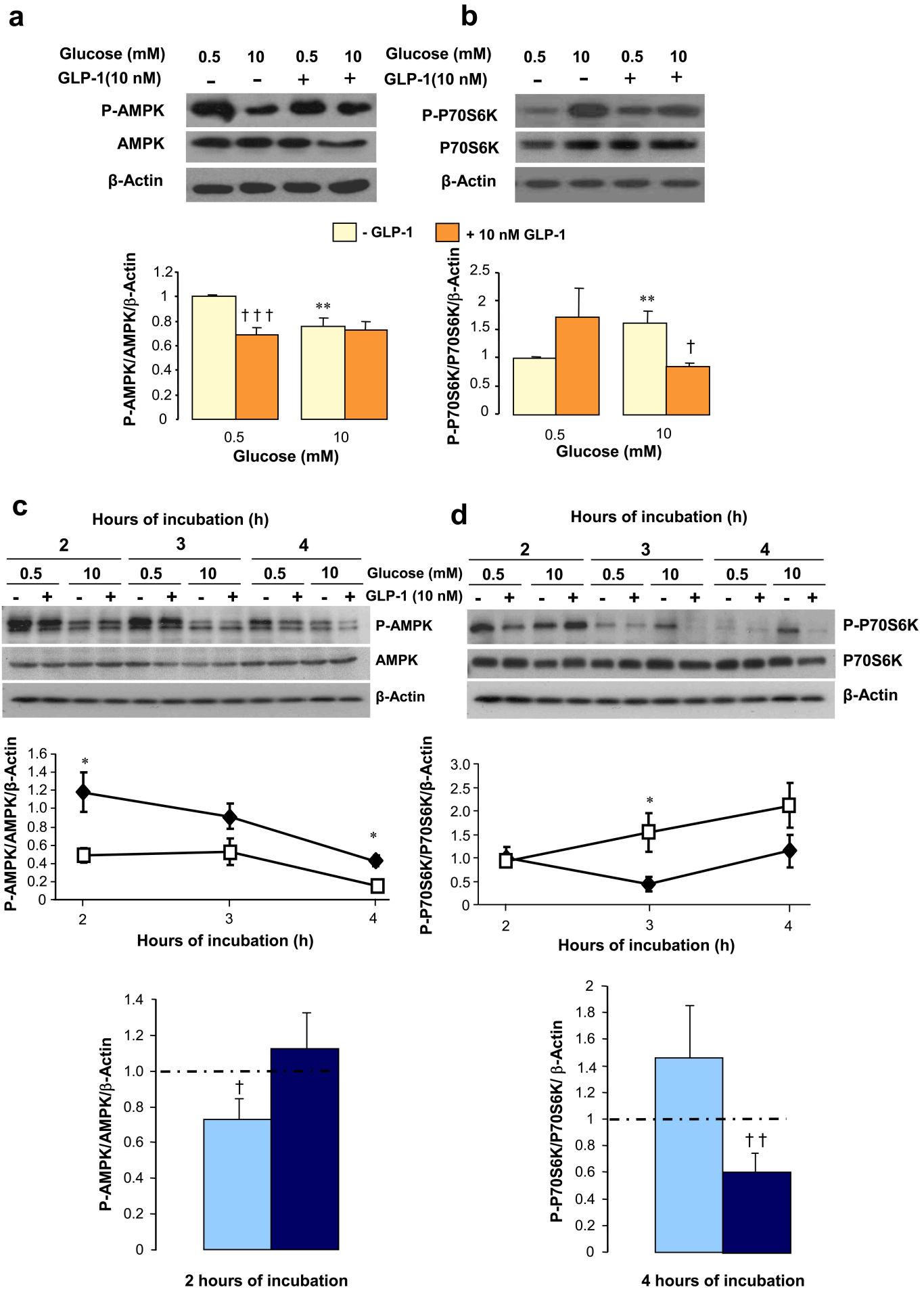

Figure 5
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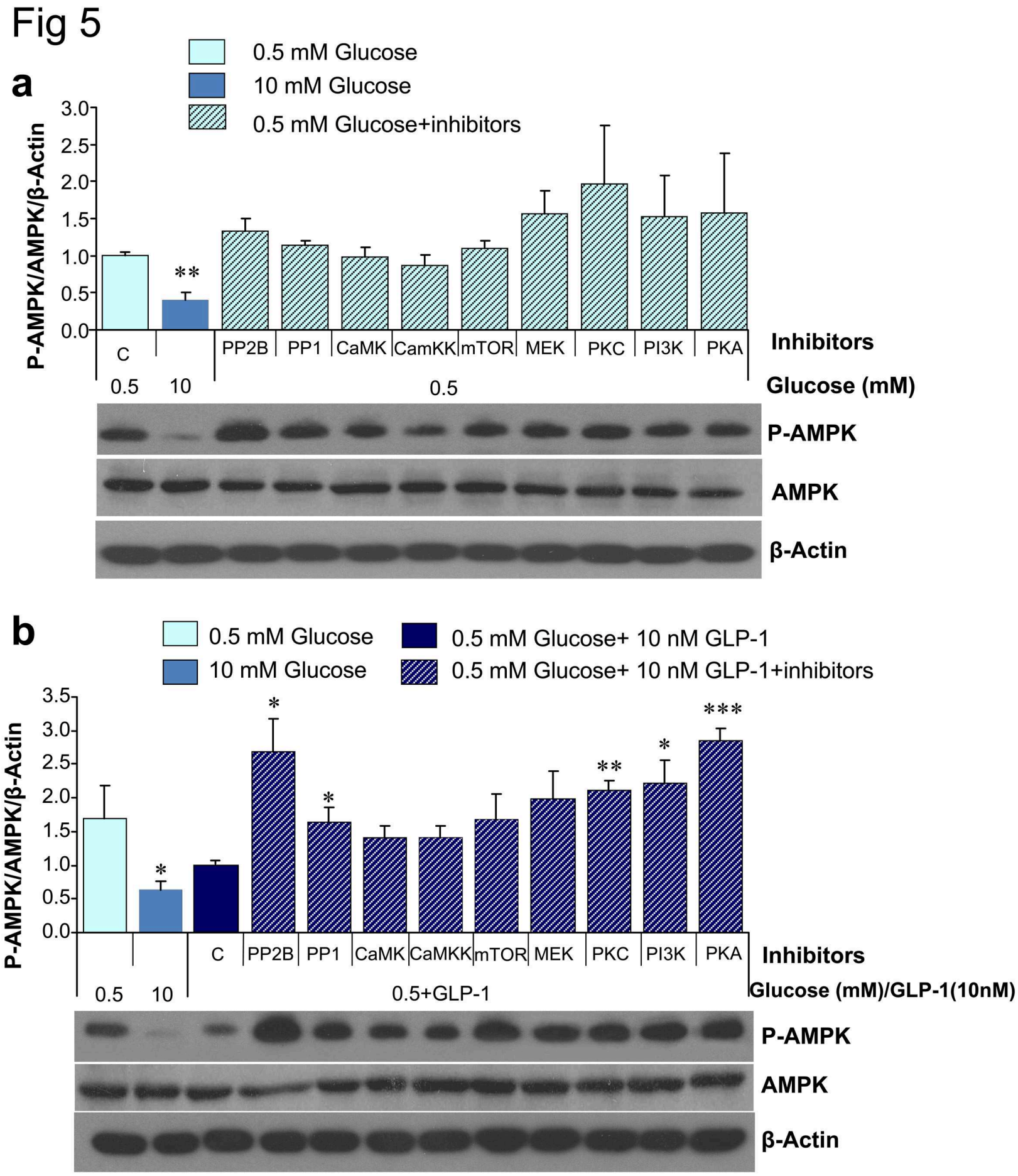

Figure 6
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Fig 6

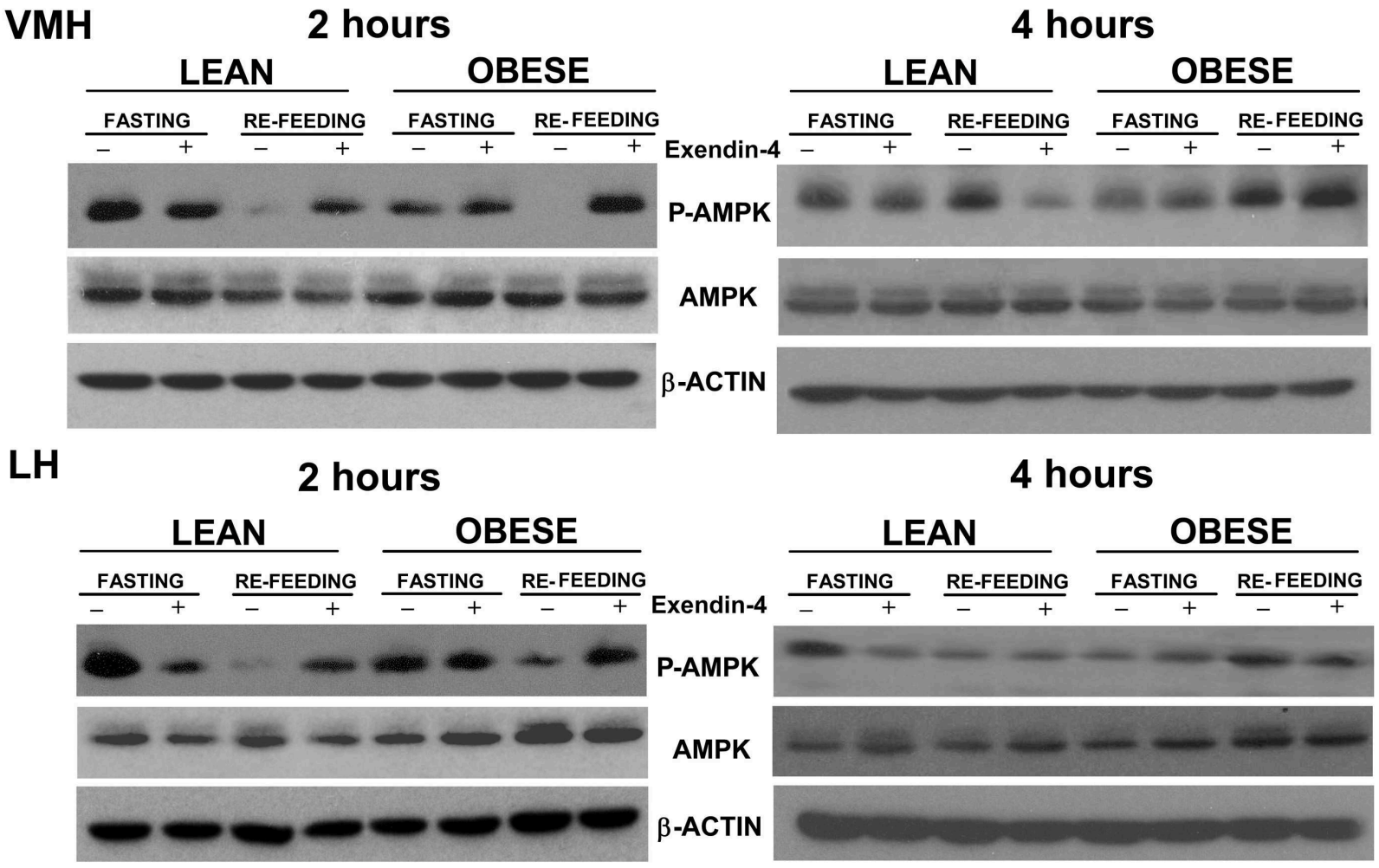

Figure 7
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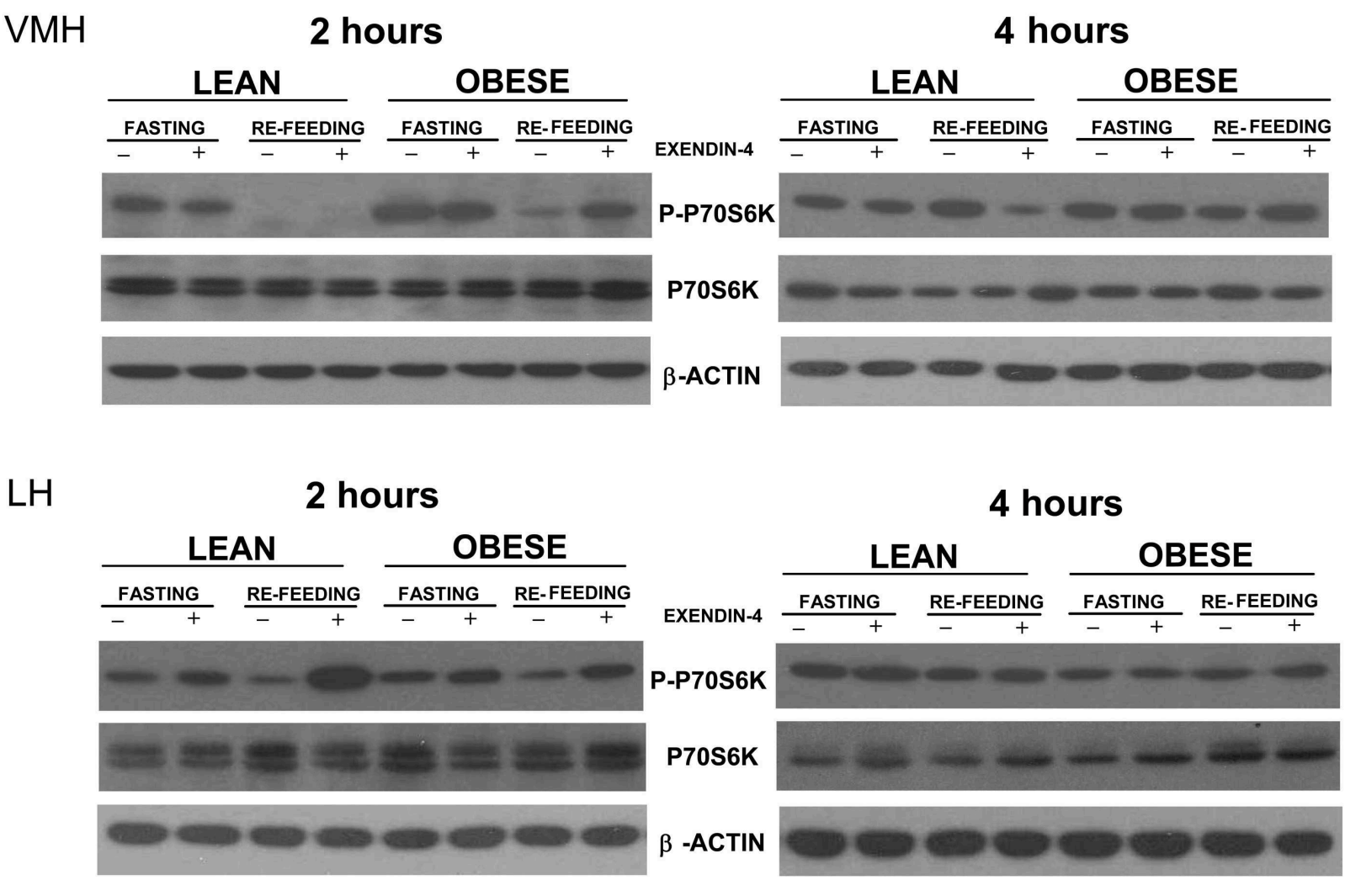
Fig 7



Online resource 1
Click here to download Table: Table1 ESM.doc

**Electronic supplementary material**

**Online Resource 1.** Primers and probes for real-time RT-PCR analysis

| Gene | Forward primer | Reverse primer | Taqman probe |
|---|---|---|---|
| *Ampk-α2* | 5´-GTGGATCGCCAAATTATGCA-3´ | 5´-GGGAGGGTGCCACAGAGAA-3´ | 5´-6-FAM-TAC AGC CTT CTT GAC ATG A-MGB-3´ |
| *18s* | | | Hs 99999901_s1 |

Online resource 2
Click here to download Table: Table 2 EMS.doc

**Electronic supplementary material**

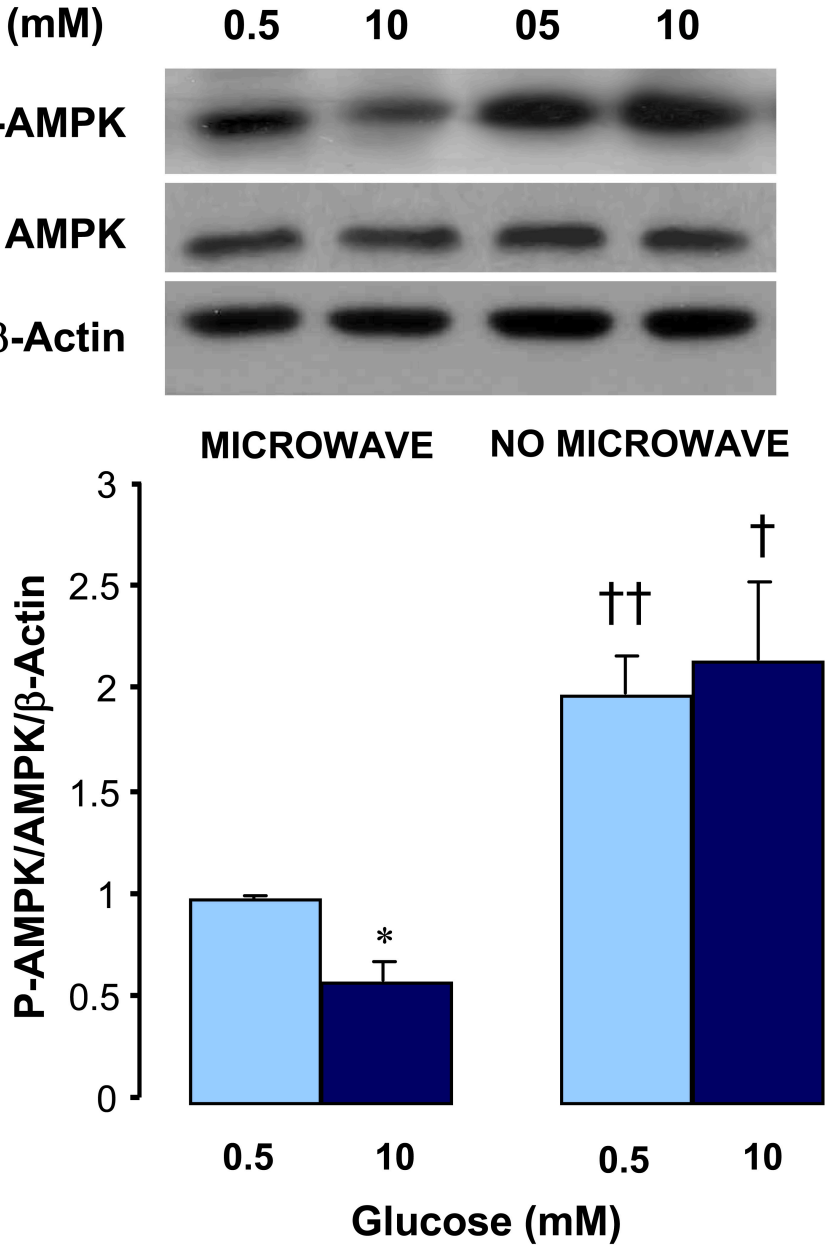
**Online Resource 2.** Antibodies and conditions used for western blot assays

| **Antibody** | **Host** | **Manufacturer** | **Dilution used** |
|---|---|---|---|
| Anti-AMPKα2 | Rabbit | Cell Signaling, Danvers, MA, USA | 1:1000 |
| Anti-phospho AMPKα (Thr172) | Rabbit | Cell Signaling, Danvers, MA, USA | 1:1000 |
| Anti-p70S6K | Rabbit | Cell Signaling, Danvers, MA, USA | 1:1000 |
| Anti-phospho p70S6K(Thr389) | Rabbit | Cell Signaling, Danvers, MA, USA | 1:1000 |
| Anti-β-Actin | Mouse | Sigma Aldrich, Madrid, Spain | 1:5000 |
| Anti-Rabbit-HRP | Goat | Millipore Iberica, Madrid, Spain | 1:5000 |
| Anti-Mouse-HRP | Goat | Bethyl Laboratories, Montgomery, USA | 1:5000 |

Online resource 3

Click here to download Figure: ON_LINE_RESOURCE_3.eps

# Electronic supplementary material




**Online Resource 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, 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 non-phosphorylated forms. The value obtained in the cells treated with 0.5 mM glucose and exposed to microwave irradiaton was taken as 1. The results are means ± SEM; $n$ = 4-5. *$P$<0.05 0.5 mM glucose vs 10 mM glucose, †$P$<0.05, ‡‡$p$<0.01 absence of microwave vs presence of microwave.

ANSWERS TO THE REVIEWERS

Reviewer #1: 1. Would be helpful to define anorexigenc and orexigenic.

In accordance with your suggestion, these terms have been defined in the revised manuscript.

2. Combine the first 3 sentences into a single paragraph.

These sentences have been included in the first paragraph of the revised manuscript.

3. Several grammatical/typographical errors need to be fixed, e.g. "knocked out" and "between these areas" on p. 5.

Thank you. Several errors have been corrected accordingly in the revised manuscript.

4. p. 6: Cell Cultures. The word "transformed" is unclear. Are the GT1-7 cells derived from mouse hypothalamic neurosecretory cells?

Yes, GT1-7 cells are neurosecretory cells. This cell line was created by Mellon et al (1990) using a targeted tumorigenesis technique by which expression of the simian virus-40 T antigen was driven by the rat GnRH 5' regulatory region to develop a murine immortalized GnRH-secreting cell line.
We have clarified this point in the Materials and Methods section of the revised manuscript.

5. Figure 2. Need to clarify the significance relative to Fig. 1.

According to your suggestion, the significance of Fig. 1 has been clarified: ***P<0.001 10 mM glucose vs 2.5 mM glucose, †††P<0.001, absence of GLP-1 vs the presence of 10 nM GLP-1

In the VH, total RNA goes down in 10 mM glucose. In Fig. 2a, phosphoAMPK goes down in 10 mM glucose. It should be clear that this is total activity and may reflect a change in total AMPK without a change in the specific activity of AMPK in the cells. In Fig. 2b, since there are no data regarding the levels of total p70S6K, it is difficult to draw a conclusion regarding the regulation of phosphorylation.

The data obtained using hypothalamic-slice explants showed a decrease in AMPK mRNA expression in LH treated for 3h with 10 mM glucose but not in VMH. However, the level of total protein remained constant during the periods of incubation used in our study. We have performed the internal control of total amount of AMPK and p70S6K protein in all the experiments, observing that the total amount of AMPK or p70S6K remained constant independently of the treatment. In the revised manuscript we have added a new panel in the Fig 2a, b , as well as in the Fig.3, 4 and 5, showing the western blots of total protein AMPK and P70S6K.

6. Figure 3. In the text, the authors state that the level of phospho-AMPK increases in Laemmli buffer. However, according to the figure legend, RIPA buffer is used. Also, the authors state that microwaving allows retention of phosphor-AMPK, but no direct comparison is made between microwaved and control cells. Also, phosho-AMPK actually goes down in 10 mM glucose (consistent with Fig. 2).

Sorry for the discordance. The cells were lysed in RIPA buffer and exposed or not to

microwave irradiation for 5 s, then Laemmli buffer was added and processed for western blot analysis. This information has been included in the legend of Fig 3. The direct comparison between microwaved and no microwaved treated cells has been included as Online Resource 3.

7. Figure 4. It is not possible to describe the higher levels of phosphorylated p70S6K as an activation without knowing the levels of total p70S6K.

In order to show all the controls in the figure, we have added a new panel in the Fig 4, which displays a western blot of total level of protein p70S6K. Those controls confirm that the total amount of p70S6K protein remained constant during the incubation time in the different conditions used in the experiment.

8. Is there significance to the fact that p79S6K is a doublet on some blots but not others?

The antibody (cat# 9202 Cell Signalling Technology) used recognizes p70S6 kinase and also p85S6 kinase, both isoforms are derived from the same gene and are identical except for 23 extra residues, which encode a nuclear localizing signal. The activity of p70S6K is controlled by multiple phosphorylation processes: phosphorylation of Thr229, Thr389, Ser411, Thr421, Ser424 and Ser371 have been identified. Phosphorylation of Thr389 correlates closely with kinase activity in vivo (Weng QP et al. J. Biol. Chem. 273, 16621-29, 1998).
We think that doublets observed correspond to different degree of p70S6K phosphorylation, which may change during the activation processes.
We appreciate very much your criticisms and suggestions. They have certainly contributed to improving the quality of the manuscript.

Reviewer #2: This manuscript describes the coordinated effects of glucose and GLP-1 on the expression and activity of AMPK and p70S6K in specific regions of the hypothalamus. They study the effects of GLP-1 on the expression and activities of AMPK and p70S6K in hypothalamic-slice explants, in GT1-7 cell line, and in both fasted and refed obese Zucker and lean control rats. The findings are an extension of what is already reported in the literature. There are modest advances described, mainly differences in the ventromedial and lateral hypothalamic areas.

Comments:

1. Neither mouse neuroblastoma N2A cells nor GT1-7 cells are appropriate models of hypothalamic feeding neurons. These cell lines are from regions not involved in energy homeostasis; thus the results described herein would not likely correlate to feeding peptidergic neurons from the hypothalamus. Appropriate models of feeding-related cell lines should be used to confirm these results.

We agree with you that these cell models can not be used exclusively for these studies. Therefore, we have used parallel models *in vivo* and *in vitro* and the results obtained with cell lines support the results obtained *in vivo*. Thus, we have made three approaches (*in vitro* experiments: cell lines and hypothalamic slices and *in vivo* experiments with Zucker rats). The *in vivo* experiments constitute the best model, however, not all the studies can be made *in vivo* and thus we have also used the *in vitro* models. The hypothalamic slices is a physiological model that allows us to work with different conditions *in vitro* with a physiological approach quite close to what happens *in vivo* in certain hypothalamic areas. This technique has been widely used for neurophysiological studies and the results published in relevant journals.
The hypothalamus has a major role in the neuroendocrine control and is involved in the regulation of physiological processes as complex as eating behaviour,

reproduction, etc. This area also contains a high cellular heterogeneity, making it impossible to use one cell line model for the analysis of complex actions such as regulation by neuropeptides (J neuroci 12, 2005, 9497-9506). Finally, there are not many hypothalamic cell lines, although there are some (J Neurosci 12: 9497-9506, 2005; Acta Pharmacol Sin 26: 976-81, 2005; The Open Neuroendocrinology Journal 3: 6-15, 2010). In addition, some of them are originated from embryonic hypothalamus, and may or may not be an accurate representation of the processes established in the adult.
Our interest in GT1-7 cells is based on previous studies where it has been demonstrated that these cells express the GLP-1 receptor and its ability to respond to changes in extracellular glucose concentration, conditions necessary to study the effects of GLP-1 in the mechanisms of response to glucose (Sanz et al. 2007; Sanz et al. 2008). It has been also reported in these cells, that the AMPK phosphorylation degree was linearly regulated by physiological levels of glucose concentrations. Additionally, changes in AgRP expression by glucose levels were similar to that observed in arcuate nucleus during fasting and re-feeding of rats (Lee K et al. Endocrinology 146: 3-10, 2004). These cells together with N2A a neuroblastoma cell line, used as a model of general neuron cells, respond to changes in the levels of extracellular glucose by modulating the activity of AMPK or p70S6K in a similar way that was observed in the *in vivo* approach used in this work. In conclusion, the results obtained from these cell lines might support the data obtained from the *in vivo* models and hypothalamic slices. In any case may be complementary with the data obtained with other experimental designs used in this work.

2. It is difficult to determine if the microwave irradiation introduces an inappropriate artifact. How do the authors know that the original results without the irradiation are not more representative of the activity in vivo? Proper positive and negative controls should be introduced with these experiments to prove that this technique is suitable.

The response in the AMPK activity to changes in the levels of extracellular glucose, has been previously described in cell lines derived from pancreatic beta-cells (Salt IP et al. Biochem J 385: 533-39, 1998) and GT1-7 (Lee K et al. Endocrinology 146: 3-10, 2004). We initially used GT1-7, N2A and one of the beta-cell lines to investigate the effect of the different glucose concentrations in the AMPK activity. We detected considerable variability from one day to the other of the experiments. We found that AMPK phosphorylation was dependent on the experimental conditions used to prepare the cell lysates. 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. Our results agree with a previous reported method of tissue preparation (Scharf et al. 2008). A new Figure with the experimental controls showing the direct comparison of the AMPK activity between both methods (with or without irradiation) has been included as Online Resource 3 in the revised manuscript. Only the cells radiated had a reproducible activation of AMPK in response to low glucose levels, in concordance to the previous results published with others cells lines such as GT1-7 or beta-cell derived cell lines.

3. Why did the authors choose a 48 h fast - this seems extreme and perhaps other pathways are activated by such a long fast.

We have initially tested the effect of a 24 h fast and 2h of re-feeding. Surprising we were nor able to reproduce the effect of fasting on the AMPK activity previously described (Kim et al. 2004; Minokoshi et al. 2004). Adittionally, Hayes MR et al. using male Sprague Dawley rats, also reported that 48h but not 24h food deprivation were necessary to increase significantly AMPK activity in hindbrain nucleus tractus

solitarius and hypothalamus (Hayes MR et al. Endocrinology 150:2175-82, 2009). A representative western blot of that test was included as "information to referee 2".

4. The levels of high glucose that were used in this study (low (0.5 mM) and high (10 mM) glucose concentrations) would not likely ever be seen in the hypothalamus. The highest levels are probably closer to 1 uM, whereas 0.5 uM would be considered normoglycemic. This should be discussed in the paper and considered carefully when assessing the results with glucose.

Silver IA and Erecinska M reported that in normoglycemic rats the extracellular glucose concentration in the brain was ~2.5 mM, at blood glucose levels of ~15 mM cerebral glucose increased to ~4.5 mM and in Zucker obese rats the levels of blood glucose may increase to ~20-25 mM. It has been also reported that ATP concentration increased markedly in a range of 1-5 mM glucose in GT1-7 and N1E-115 neuroblastoma cells and the concentrations above of 5 mM glucose ATP levels were maintained stable (Lee K et al. Endocrinology 146: 3-10, 2004). These data were used to choose the glucose concentrations used in our study.
We have discussed in the revised manuscript, the glucose concentrations used to reproduce in the cell lines the results obtained in experiments in vivo.

5. Further the cell lines used were cultured in 25 uM glucose which probably represents hyperglycemia or possibly glucotoxicity. This should also be considered in the discussion.

Yes the cell lines were cultured in DMEM containing 25 mM glucose. These conditions are recommended to grow these cell lines and have been used in previous published studies (Mellon,P. Neuron 5:1-10, 1990; Lee K et al. Endocrinology 146: 3-10, 2004; Sanz et al. 2008). The Neurobasal medium used to grow neurons specifically also contains 25 mM glucose. There are studies of toxicity where N2A maintained in DMEM 4.5 g/l glucose were used as control (Manzoni et al. PLoS ONE: 6 (9), 2011). Using that medium, we observed that both cells lines (N2A and GT1-7) grown normally with no apparent signs of glucotoxicity. Any way previous to stimulate the cells with different glucose concentrations, all the cells were grown 2 hours in 0.5 mM glucose in order to initiate the stimulation in more physiological conditions.

6. The authors use total AMPK and P70S6K as normailization factors for their western blots in Figs 4, 6, 7. This should also be used in Figs 2, 3, 5. It is unclear why this was not done.

In order to show all the controls in the figures, we have added new panels in the Fig 2, 3, 4 and 5 which displays a western blot of total level of protein AMPK and p70S6K. Those controls confirm that the total amount of AMPK and p70S6K protein remained constant during the incubation time under the different conditions used in these experiments. We had not included these data in the original manuscript because no changes were observed in any case with the different treatments used.

7. The controls for the inhibitors alone are not included. These are absolutely critical, as many have basal effects on kinase activity.

Actually the Figure 5 included these controls, although the complexity of the data shown in this figure may lead to misinterpretation. We apologize for this induced confusion.
The control experiment showing the basal effect produced by adding only inhibitors are included in the panel (a) of Figure 5 (0.5mM glucose plus inhibitors alone). The first two lines in the western blot correspond to the levels of AMPK at low or high

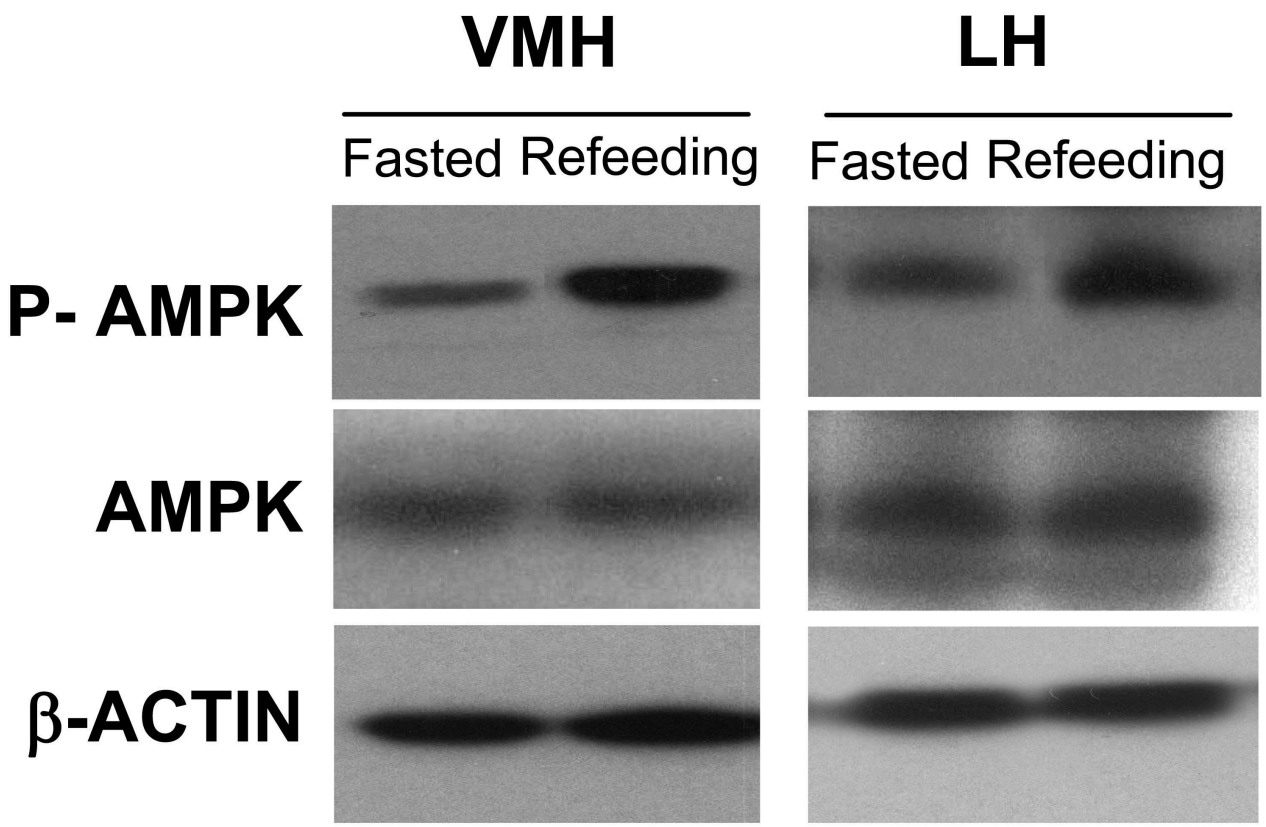
glucose in the absence of inhibitors and the hatched bars represent the basal effect of the different inhibitors on AMPK activation at low glucose levels. Our results showed that the differences were not statistically significant in the presence of any of inhibitors used. However, the panel (b) shows the effect of the addition of GLP-1 to the cells treated in the same conditions that in the panel (a).

While the results described add some additional knowledge to what is currently understood about GLP-1 action in the hypothalamus, a more mechanistic approach would have been appreciated.

The GLP-1 receptor agonist exendin-4 is one of the antidiabetogenic agents used in clinical practice (Niswender 2010) and is a long-acting agonist that also produces weight loss (Blonde et al. 2006; Buse et al. 2009; Montanya and Sesti 2009). However, it has not been established before a direct relationship between the effect of this anorexigenic peptide and some of the metabolic sensors that are involved in the regulation of feeding behaviour. There is also limited information on how hypothalamic metabolic sensors respond in obese individuals and we believe this work is needed to open the field to further study of the mechanisms involved in these functions.
We appreciate very much your criticisms and suggestions. They have certainly contributed to improving the quality of the manuscript.

Click here to download Figure: INFORMATION TO REFEREE 2.eps

# Information to referee 2




**Effect of 24 h food deprivation on AMPK activity.**
Animals were food-deprived for 24 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-AMPK (Thr-172) (P-AMPK) and total AMPK (AMPK) were analyzed by western blot. The blots were reprobed for β-Actin.