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ScienceDirect
Metabolism Clinical and Experimental 57 (2008) 40–48
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

# Leptin but not neuropeptide Y up-regulated glucagon-like peptide 1 receptor expression in GT1-7 cells and rat hypothalamic slices

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Received 26 October 2006; accepted 3 August 2007

**Abstract**

In an attempt to gain better insight into the central effects of glucagon-like peptide (GLP-1), we studied the action of glucose and of regulatory peptides on the expression of its receptor (GLP-1R) in hypothalamic GT1-7 cells and in ventromedial (VMH) and lateral (LH) rat hypothalamus slices. The promoter activity of GLP-1R in transfected GT1-7 cells increased with leptin, whereas neuropeptide Y (NPY) did not modify it. Interestingly, when cells were incubated with both NPY and leptin, NPY blocked the stimulating effect of leptin. The effects of leptin and NPY were also confirmed at messenger RNA levels. In hypothalamic slices, GLP-1R messenger RNA levels increased at higher glucose concentrations in the VMH. In addition, leptin exerted a stimulating effect; and NPY did not modify receptor expression. By contrast, in the LH, the opposite effects were found for those parameters, except at 20 mmol/L glucose. These findings suggest that the stimulating effect of leptin on GLP-1R expression, with no changes in NPY-induced activity, could enhance the anorexic actions generated through this receptor. In addition, the different responses of the VMH and LH may be related to specific functions of these structures, as already known in vivo, highlighting the interest of hypothalamic slices for this kind of study.


## 1. Introduction

Proglucagon gene expression in gut and brain gives rise to several molecules [1], glucagon-like peptide 1 (GLP-1) (7-36) amide being one of the products generated from the C-terminal portion, with greater biological activity in peripheral tissues and the central nervous system. GLP-1(7-36) amide stimulates insulin secretion in a glucose-dependent manner [2], and it inhibits gastric emptying [3]. This peptide also increases arterial blood pressure and heart rate in rats [4], and it increases surfactant secretion by rat [5] and human [6] type II pneumocytes. These effects are achieved through the GLP-1 receptor (GLP-1R), which has been cloned and sequenced, with the finding that the sequences deduced are the same in both pancreatic islets and the brain [7-11].

The synthesis of GLP-1(7-36) amide and the presence of a high GLP-1R density in neurons of the same brain areas may be responsible for the reported central effects of GLP-1 (7-36)amide on the selective release of neurotransmitters [12], appetite, and fluid homeostasis [13,14]. Interestingly, in the brains of humans and experimental animals, GLP-1(7-36) amide serves as a signal to reduce food intake, acting as an anorexigenic peptide. Furthermore, the coexpression of GLP-1 receptors, glucokinase, and the glucose transporter (GLUT) isoform GLUT-2 in hypothalamic cells involved in feeding behavior suggests that they might play a role in glucose sensing [9,14,15]. The first indications that the hypothalamus plays a major role in feeding behavior and energy homeostasis were reported 60 years ago after brain lesioning and stimulation studies. Taking advantage of these early observations, such as the fact that electrical stimulation of the ventromedial hypothalamus (VMH) suppresses food intake and that bilateral lesions of these structures induce hyperphagia and obesity, the VMH was named the *satiety center*, whereas alterations to the lateral hypothalamic area (LH) induced the opposite set of responses and LH was hence called the *hunger center*. These brain areas use autonomic efferent nerves to modulate glucose homeostasis in the liver and endocrine pancreas; and they may be altered by metabolic signals, such as changes to the electrical activity of neurons by direct application of glucose or by modification of blood glucose levels [16,17]. In fact, 2 kinds of glucose sensor neurons have been identified [18,19]. First, there are glucose-excited neurons, which are present mainly in the VMH and which

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doi:10.1016/j.metabol.2007.08.005

are excited by increased glucose levels in the extracellular space, with alterations to their firing rates. By contrast, in the LH, there are a significant number of glucose-inhibited neurons; and these are excited by decreases in glucose levels in the extracellular space [18,20].

Current evidence points to the existence of a specific subpopulation of neurons involved in energy homeostasis, located in the so-called *satiety and hunger centers*, that are neuronal pathways with orexigenic and anorexigenic peptides that generate integrated responses to afferent stimuli related to modifications in metabolites or in fuel storage. GLP-1(7-36)amide may be considered an anorexigenic peptide [13,14] able to induce central effects that contribute to feeding behavior, supported at least in part by the effects of anorexigenic and orexigenic peptides. Accordingly, it seems of interest to determine the factors that control GLP-1R expression in the brain and the role in this of the glucose levels present in the extracellular space and/or regulatory peptides. To accomplish these aims, we used cells of hypothalamic origin such as GT1-7 to study the transcriptional regulation of the GLP-1R gene in cells transiently transfected by vectors containing the promoter of GLP-1R fused to the luciferase gene and also short-term cultures of hypothalamic slices from adult rats. There are experimental evidences that GT1-7 cells are glucose-sensing cells [21,22] able to respond to glucose deprivation or high glucose levels. They represent a model for studying the transcriptional regulation because the transfection method cannot be applied to other systems, including hypothalamic slices. However, immortalized cell lines are limited in studies of transcriptional activity because they are not located within a complex neuronal network. Thus, a further step was the use of rat hypothalamic slices, which represent an experimental design of physiological relevance because they preserve some of the tissue architecture and the functional connections involved in feeding behavior. The findings obtained may open an experimental approach to study the interacting effects of these regulatory peptides in hypothalamic neurons as well as in hypothalamic slices.

## 2. Methods

### 2.1. Experimental animals

Male Wistar rats weighing 150 to 175 g were fed ad libitum with a standard pellet diet and housed at a constant temperature (21°C) on a 12-hour light-dark cycle with lights on at 8:00 AM. Rats were killed by decapitation, and the whole brain was rapidly removed and placed in ice-cold medium. All procedures were carried out according to the European Union ethical regulations for animal research.

### 2.2. Cell cultures and GLP-1R promoter activity assays

The GT1-7 cell line (generously provided by Prof P Mellon, San Diego, CA), transformed from mouse hypothalamic neurosecretory cells [21], is a good tool for neuronal studies [23]. The GT1-7 cells were maintained in Dulbecco modified Eagle medium (Life Technologies, Barcelona, Spain) containing 25 mmol/L glucose, 10% fetal bovine serum (FBS) (Biomedia, Boussens, France), 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 2 mmol/L glutamine (Biomedia). The wild-type reporter gene construct (generously provided by Dr Lankat-Buttgereit, Marburg, Germany) was created by cloning 2958 base pairs (bp) of the human GLP-1R promoter (−2922 to +36) into the promoterless luciferase vector pGL2 Basic (Promega, Madison, WI). Transfection was performed using calcium phosphate precipitates containing 3 $\mu$g of luciferase expression vector with the GLP-1R promoter and 1 $\mu$g of the internal control plasmid pCMV-$\beta$-galactosidase for each well of a 12-multiwell plate. The GT1-7 cells were incubated with calcium phosphate precipitates for 14 to 16 hours and then rinsed and kept in fresh medium for 24 hours. After this, the cells were incubated overnight in the presence of medium containing 2% FBS and 5.5 mmol/L glucose. The medium was then removed and replaced by Dulbecco modified Eagle medium containing 2% FBS and different glucose and/or peptide concentrations. After 5 hours of treatment, the cells were lysed; and luciferase activity was determined with a luminometer (BG-P luminometer; GEM Biomedical, Hamden, CT). Luciferase activity levels, normalized with $\beta$-galactosidase activities, are expressed as percentages of control (5.5 mmol/L glucose) values.

### 2.3. Procedure for hypothalamic slice explant culture

Hypothalamic slice explant cultures were performed as described previously [24,25]. Male Wistar rats were killed by decapitation; and the brains were quickly removed and immersed in cold (4°C) MEM medium containing 25 mmol/L N-[2-hydroxyethyl]piperazine-N′-[2-ethalnesulfonic acid] (HEPES), 20% heat-inactivated horse serum, 4 mmol/L glutamine, 6.5 mg/mL glucose, and 100 U/mL penicillin-streptomycin. After this, the hypothalamus was removed from the brain and sectioned at 300-$\mu$m thickness on a Mcllwain tissue chopper (Mickle Laboratory Engineering, Surrey, United Kingdom). Coronal slices were separated and placed in MEM supplemented with 25 mmol/L HEPES and Hank salt (Life Technologies) enriched with the above components. In an attempt to stabilize the cultures, hypothalamic slices were cultured at 37°C in an atmosphere of air and $CO_2$ (95%:5%, respectively) in this medium (MEM, 25 mmol/L HEPES with Hank salt, 20% heat-inactivated horse serum, 4 mmol/L glutamine, 36 mmol/L glucose, and 100 U/mL penicillin-streptomycin) for 5 hours. Afterward, the hypothalamic slices were transferred to a medium containing 2% FBS and 5.5 mmol/L glucose and incubated for 16 hours. The cell viability of the hypothalamic slices was determined by the addition of 0.5 $\mu$g/mL of propidium iodide to the culture medium or by incubating the slices in a solution containing 1 mL of a trypan blue solution (0.8 mmol/L in PBS) and 1 mL of culture medium. After at least 15 minutes of incubation, propidium staining was

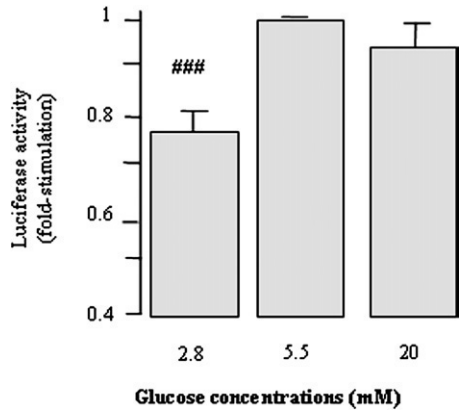


Fig. 1. Effects of glucose on GLP-1R promoter activity in GT1-7 cells. The GT1-7 cells were transiently transfected with a luciferase reporter plasmid containing GLP-1R promoter. Cells were then incubated with 2.8, 5.5, or 20 mmol/L glucose. Data are presented as fold increase of GLP-1R-luciferase activity (ratio of luciferase to $\beta$-galactosidase activity) relative to 5.5 mmol/L–glucose treatment ± SEM. The experiment was repeated at least 7 times in duplicate. ###$P \leq .001$ (2.8 mmol/L glucose vs 5.5 or 20 mmol/L glucose).

assessed with an inverted fluorescence microscope using a standard rhodamine filter set. In the case of trypan blue, cell viability was determined under a light microscope 5 minutes after incubation with the stain.

To examine the effects of different glucose concentrations and/or peptides on GLP-1R messenger RNA (mRNA) levels in hypothalamic slices, these were incubated with 0.5, 1, 2.8, 5.5, or 20 mmol/L glucose and/or peptides at the indicated concentrations for 5 hours. Optimal concentrations and times of incubation were selected after preliminary assays of endogenous GLP-1R gene expression either in GT1-7 cells or hypothalamic slices (data not shown) using dose- and time-response curves. 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 of Paxinos and Watson [26]. To map the functional activity of VMH and LH, the expression of the immediate early gene *c-fos* was determined [27,28]. Thus, to validate the functional activity in these 2 areas, c-Fos protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and determined by immunodetection in hypothalamic slices incubated for 3 hours in the presence of low and high (1 and 20 mmol/L) glucose concentrations [29].

### *2.4. RNA isolation and reverse transcriptase polymerase chain reaction*

Total RNA from culture cells and from areas of rat hypothalamic slices were extracted with TRIZOL (Life Technologies). The purity of the RNA was assayed by electrophoresis in an agarose gel. One microgram of total RNA was used to obtain complementary DNA (cDNA) (RETROscript; Ambion, Austin, TX). Four microliters of 1:10 diluted cDNA was used as template for the polymerase chain reaction (PCR) with the SYB Green PCR Master Mix (Applied Biosystem, Foster City, CA) in a real-time thermocycler (ABI PRISM 7300; Applied Biosystem) following the manufacturer's instructions. In some cases, we confirmed the results by performing the real-time PCR with Taqman probes (GLP-1R: ref Rn00562406_m1; 18S: ref. Hs99999901_s1) or by semiquantitative PCR. Primer sequences were designed using Primer Express software (Applied Biosystems). The real-time PCR parameters were validated, and the best conditions and primer set were chosen to obtain the highest specificity and efficiency on PCR results. In this way, 2 pairs of primers for GLP-1R and a pair for $\beta$-actin ones were tested at different combinations of primers concentrations (50, 300, and 900 nmol/L). For the best real-time PCR efficiency (92%), with no contaminating products or primer dimers present in the reaction as indicated by the melting curves, the primers finally selected for the GLP-1R amplification were 5′-CCGGGTCATCTG-CATCGT-3′ (300 nmol/L) and 5′-AGTCTGCATTT-GATGTCGGTCTT-3′ (300 nmol/L), rendering a single amplicon product of 72 bp. To control the differences in initial RNA levels and tube-to-tube variations in real-time

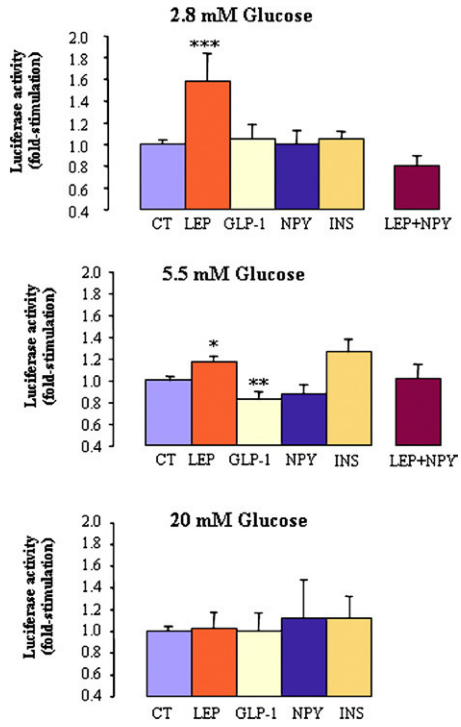


Fig. 2. Effects of glucose and regulatory peptides on GLP-1R promoter activity in GT1-7 cells. The GT1-7 cells were transiently transfected with a luciferase reporter plasmid containing GLP-1R promoter. Cells were then incubated with 2.8, 5.5, and 20 mmol/L glucose in the absence or presence of different peptides: 10 nmol/L leptin, 10 nmol/L GLP-1, 100 nmol/L NPY, 10 nmol/L insulin, or a combination of 10 nmol/L leptin plus 100 nmol/L NPY. Values are expressed as fold stimulation of luciferase activity (ratio of luciferase to $\beta$-galactosidase activity) relative to control. Data represent the means ± SEM of data from at least 7 independent experiments developed in duplicate. *$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$ (incubated with peptides vs control). CT indicates control; LEP, leptin; INS, insulin.

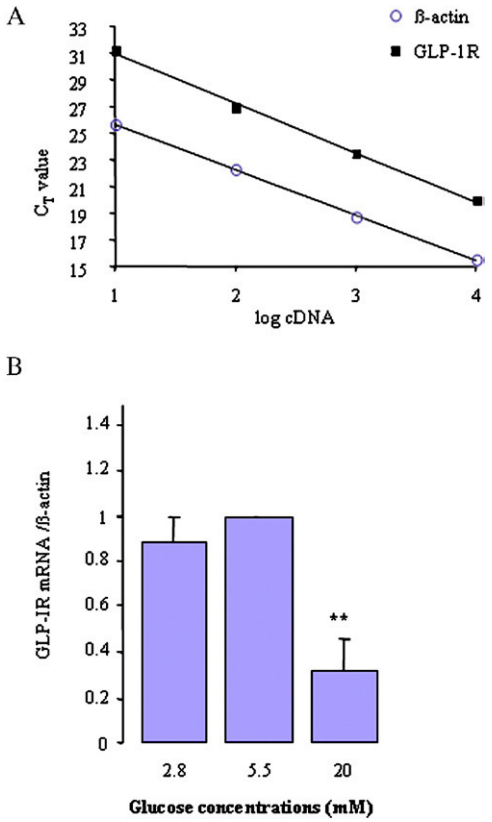


Fig. 3. Effects of glucose concentrations on the GLP-1R mRNA levels in GT1-7 cells. The GT1-7 cells were incubated for 5 hours in the presence of different glucose concentrations (2.8, 5.5, and 20 mmol/L). Total RNA was extracted with TRIZOL, and 1 $\mu$g was transcripted to cDNA. The cDNA was used as a template for the real-time PCR. A, Representative standard curves for GLP-1R and $\beta$-actin. The correlation coefficient for standard curves ranged from 0.995 to 0.999, suggesting that the quantification was accurate ($C_T$ 40 means that there was no detectable signal after 40 cycles of PCR amplifications). B, The bars represent the GLP-1R mRNA fold increase expression after normalization by $\beta$-actin and as compared with the 5.5 mmol/L–glucose values. Each value is the mean ± SEM of duplicates of at least 3 independent experiments. **$P \leq .01$ (20 vs 5.5 mmol/L glucose).

PCR, a primer pair for $\beta$-actin (300 nmol/L) was included in each PCR amplification. The control $\beta$-actin amplification primers were 5′-AGGCCAACCGTGAAAAGATG-3′ and 5′-CACAGCCTGGATGGCTACGTA-3′, which resulted in an amplicon of 80 bp. The conditions used were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Using the manufacturer's software, a threshold above the noise was chosen; and the cycle number at which fluorescence exceeded the threshold ($C_T$) was determined for each well. In each real-time PCR assay, a standard curve was generated by 10-fold serial dilutions in water of the cDNA samples. An example is shown in Fig. 3, where the slopes were around 3.03 and 3.23 for GLP-1R and $\beta$-actin, respectively.

The mean $C_T$ value for each cDNA sample was expressed as an arbitrary value relative to the standard curve after linear regression analysis. Data were normalized with $\beta$-actin values and were expressed as arbitrary units, considering the results obtained after the incubation of the biological samples with 5.5 mmol/L glucose alone as a value of "1".

### 2.5. Statistical analysis

All values are presented as mean ± SEM. Comparisons among groups were made using analysis of variance. $P \leq .05$ was considered statistically significant.

## 3. Results

### 3.1. Effects of glucose and/or regulatory peptides on GLP-1R promoter activity in GT1-7 cells

The action of glucose and of regulatory peptides on GLP-1R promoter activity was tested in GT1-7 cells transfected with the GLP-1R promoter fused to the luciferase gene. The GLP-1R promoter activity was higher at 5.5 and 20 mmol/L than at 2.8 mmol/L glucose (Figs. 1 and 2). In addition, the regulatory peptides studied modulated GLP-1R gene transcription; such was the case of leptin, which induced a significant increase in promoter activity (Fig. 2), with this effect being higher at lower glucose concentrations. The only peptide that inhibited transcriptional activity was GLP-1, which significantly reduced promoter activity at 5.5 mmol/L glucose (Fig. 2). By contrast, neuropeptide Y (NPY), the main orexigenic peptide, and insulin did not induce promoter activity (Fig. 2). Interestingly, when the cells were incubated with both leptin and NPY, the latter blocked the stimulating effect of leptin (from 1.57 ± 0.26 relative luciferase activity when cells were incubated with 2.8 mmol/L glucose and 10 nmol/L leptin to 1.02 ± 0.13 relative luciferase activity when they were incubated with the same components plus 100 nmol/L NPY).

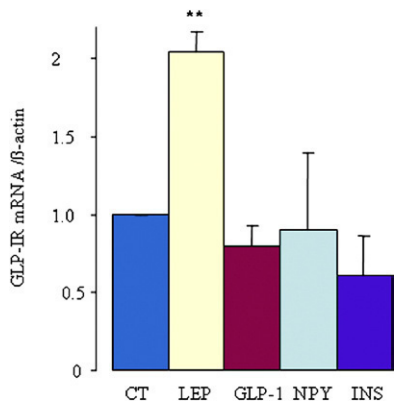


Fig. 4. Effects of regulatory peptides on the GLP-1R mRNA levels in GT1-7 cells. The GT1-7 cells were incubated for 5 hours in the presence of 5.5 mmol/L glucose (CT) and the indicated peptides: 10 nmol/L LEP, 10 nmol/L GLP-1, 100 nmol/L NPY, 10 nmol/L INS. Total RNA was extracted with TRIZOL, and 1 $\mu$g was transcripted to cDNA. The cDNA was used as a template for the real-time PCR. The bars represent the relative abundance of GLP-1R mRNA after normalization by $\beta$-actin; comparisons were done with the 5.5 mmol/L–glucose values. Each value is the mean ± SEM of at least 3 independent experiments. **$P \leq .01$ (LEP vs CT).

### 3.2. Effects of glucose and/or regulatory peptides on GLP-1R mRNA levels in GT1-7 cells

The GLP-1R mRNA levels were modified by glucose concentrations and by leptin (Figs. 3 and 4). The real-time PCR results revealed that 20 mmol/L glucose produced a significant reduction in the expression of GLP-1R as compared with the values obtained at 2.8 and 5.5 mmol/L glucose (Fig. 3). When the effect of the peptides was studied at 5.5 mmol/L glucose, leptin showed the greatest activity on GLP-1R expression (Fig. 4), increasing the mRNA levels by 2-fold with respect to control conditions. In contrast, GLP-1, NPY, and insulin had no effect (Fig. 4).

### 3.3. Effects of glucose and/or regulatory peptides on the expression of GLP-1R in hypothalamic slice explants

In an attempt to gain further physiological insight into the effects of glucose and/or regulatory peptides on GLP-1R expression, we developed an experimental design with hypothalamic slices in which receptor expression was studied in the VMH and the LH areas by real-time PCR. These areas have antagonist effects on the control of food intake and on the functioning of the glucose sensor. Although most authors have used hypothalamic slices from 7-day-old rat pups for long-term cultures, we preferred to use short-term cultures of tissue slices from adult rats because, at this age, the whole of the neuronal network related to feeding behavior and the interactions of orexigenic and anorexigenic peptides with their receptors have already been established, which may offer a better physiological design. Cell viability in the hypothalamic slices was tested with propidium iodide or trypan blue immediately after the slices had been obtained from the whole hypothalamus and 16 hours later, when the different experiments were done. A minor degree of propidium or trypan blue staining at the face of tissue cut was observed after hypothalamus sectioning, but this did not increase 16 hours later with the culture of the tissue specimens.

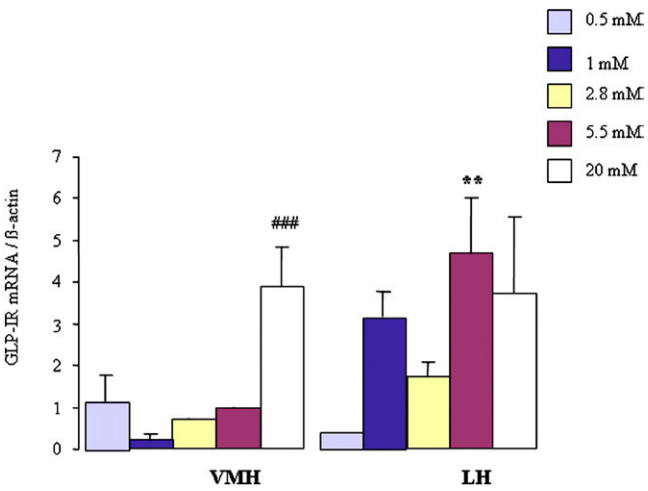


Fig. 5. Effects of glucose concentrations on GLP-1R mRNA levels as determined by real-time reverse transcriptase (RT)–PCR in hypothalamic slice explants. The hypothalamic slice explant cultures were incubated for 5 hours in a medium that contained 0.5, 1, 2.8, 5.5, or 20 mmol/L glucose. The VMH and LH areas were dissected from the hypothalamic slices after the incubation period, and total RNA was extracted for RT and real-time PCRs. The bars represent the relative abundance of GLP-1R mRNA after normalization by $\beta$-actin (1 = VMH at 5.5 mmol/L glucose). Each value is the mean ± SEM of at least 3 independent experiments. $^{**}P \leq .01$ (VMH vs LH); $^{###}P \leq .001$ (2.8 or 5.5 mmol/L glucose VMH vs 20 mmol/L glucose VMH).

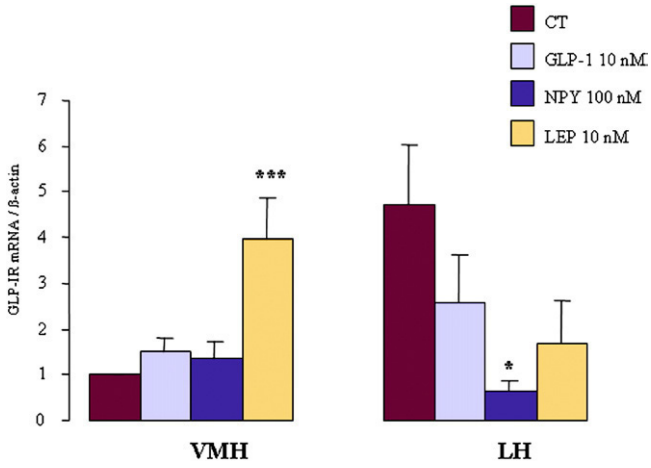


Fig. 6. Effects of regulatory peptides on GLP-1R mRNA levels as determined by real-time RT-PCR in hypothalamic slice explants. The hypothalamic slice explant cultures were incubated for 5 hours in a medium that contained 5.5 mmol/L glucose (CT) and different peptides at the indicated concentrations. The VMH and LH nuclei were dissected from the hypothalamic slices, and total RNA was extracted to make the RT and real-time PCRs. The bars represent the relative abundance of GLP-1R mRNA after normalization by $\beta$-actin (1 = values obtained in VMH at 5.5 mmol/L). Each value is the mean ± SEM of $3 \leq n \leq 8$ independent experiments. $^{***}P \leq .001$ (CT vs incubated with peptide).

Because the expression of the immediate early gene product, c-Fos, is an indicator of neuronal activity, this procedure has been used in many studies [27,28]. Neuronal functional activity was tested in VMH and LH areas by incubation of hypothalamic slices with different glucose concentrations; after the isolation of these areas, we determined c-Fos protein levels. These protein levels were high in the VMH and lower in the LH in response to high glucose concentrations (20 mmol/L), whereas 1 mmol/L glucose produced a significant increase in LH as compared with VMH [29].

The effects of glucose variations in the extracellular space on GLP-1R mRNA levels were area-specific. Thus, the increase in glucose concentration from 5.5 to 20 mmol/L produced a 4-fold increase in GLP-1R expression in the VMH but did not elicit any change in the LH (Fig. 5). However, the decrease in glucose levels from 5.5 to 2.8 mmol/L reduced the expression of the receptor only in LH and not in VMH. Obviously, glucose concentrations produce a distinctive response in the GLP-1R mRNA levels of VMH and LH. Thus, whereas at 0.5 or 20 mmol/L

glucose, the GLP-1R mRNA levels were similar in both areas, at 1, 2.8, and 5.5 mmol/L glucose, there were higher levels of GLP-1R expression in LH than in VMH (Fig. 5). Furthermore, at 5.5 mmol/L glucose, in the VMH, GLP-1R mRNA expression was significantly increased by leptin; but in LH, this effect was not observed (Fig. 6). No changes were found when GLP-1 or NPY were added to the incubation medium in VMH area. However, in the LH, where the GLP-1R expression is higher than in VMH at 5.5 mmol/L glucose, none of the regulatory peptides cited increased GLP-1R mRNA expression. Even NPY reduced significantly it, this being the peptide with the strongest effect.

## 4. Discussion

A complex network of neurons containing orexigenic and anorexigenic peptides and metabolic sensors interacting with one another in the hypothalamus enables the control of metabolic homeostasis and the nutritional state of the organism. Because the expression of the GLP-1 receptor may contribute to this process because of the anorexigenic properties of its ligand, we designed an experimental model to study its activity in response to regulatory peptides and to the level of glucose in the extracellular space mainly in the VMH and LH areas because they are implicated in feeding behavior and contain significant amounts of glucose sensors. Based on normal feeding in the GLP-1 receptor knockout mouse, the involvement of GLP-1 neurons in the anorexic actions of this peptide has been questioned. This apparent paradox may be explained because there are many anorexigenic peptides that may act either alone or through leptin or GLP-1 receptors in the control of feeding behavior, up to a point at which no significant alterations are observed when one of these peptides is missing. To study the transcriptional regulation of the GLP-1R gene, we used the GT1-7 cell line, of hypothalamic origin, transiently transfected with vectors containing the promoter of GLP-1R fused to the luciferase gene. The cell line represents an interesting model for studying the transcriptional regulation of GLP-1R gene because the transfection method cannot be applied to other systems, including hypothalamic slices. Originally, GT1-7 cells were used because they exhibited a cell physiology that resembles the luteinizing hormone–releasing hormone neuronal function in vivo, which might suggest that they do not represent a good model to study the effects of anorexigenic and orexigenic peptides. However, in the last years, GT1-7 cells have been used in many studies implicated in feeding behavior. Thus, the expression of feeding-related peptide receptor mRNAs in GT1-7 cells and the role of leptin and orexins in the control of GnRH secretion have been described [30]. In addition, there are experimental evidences indicating that GT1-7 are glucose-sensing cells [22,21,29] that are able to respond to glucose deprivation or high glucose levels, have endogenous GLP-1R expression [30], and respond to GLP-1 treatment [29,31]. Recently, we have validated the GT1-7 cells as a model to study the effects of glucose and of anorexigenic and orexigenic peptides on the transcription expression and catalytic activities of glucokinase [29], which highlights also the interest in these cells to investigate other neuronal functions not directly related with the luteinizing hormone–releasing hormone physiology, but rather with regulatory peptides implicated in feeding behavior. These facts reinforce the importance of GT1-7 cells mainly because, with a more physiological model such as hypothalamic slices, we obtained the same results with these cells on the stimulation of GLP-1R expression by leptin and the unresponsiveness to NPY. Furthermore, it is necessary to keep in mind that, although these cells offer some advantages, they constitute a limited experimental model because this tumor-derived cell line may not fully represent the hypothalamic physiology. To validate the finding with GT1-7 cells, we used a more physiological model such as rat hypothalamic slices, obtaining with both systems the same results. The GLP-1R gene transcription was modulated by changes in glucose concentrations, but the effect in the expression of GLP-1R mRNA was different. An explanation for this paradox may be due to the existence of different regulatory sequences in the human promoter used to study the transcriptional regulation as compared with the mouse promoter present in the endogenous GLP-1 receptors of GT1-7 cells. In addition, the presence of leptin significantly increased and NPY did not change the expression of GLP-1R gene, both at gene transcription level and at mRNA expression.

The response of GT1-7 cells to regulatory peptides and glucose may not reflect the true biological responses of the hypothalamus to these stimuli because of the complexity of brain tissue architecture and the relationship of regulatory peptides and their receptors at different locations in the brain. Taking this idea into consideration, we used hypothalamic slices that were incubated in the presence of different stimuli, after which the VMH and LH were isolated to determine the GLP-1R mRNA levels. We selected these 2 areas because they are considered the areas that control feeding behavior and they contain the 2 main types of glucose-sensor cells. Our findings indicating the distinctive c-Fos expression in response to different glucose concentrations [29] indicate that these hypothalamic slices kept in vitro the functional activity found in the whole animal [32]. Our results also indicate that glucose induces a different pattern of GLP-1R expression between GT1-7 cells and hypothalamic slices, and the differences were also evident between the VMH and LH. Thus, whereas the expressions of GLP-1R were similar in both areas at high concentrations of glucose (20 mmol/L), the responses at lower glucose concentrations were significantly different. These findings may reflect a distinctive response to changes in glucose concentrations of neurons located in the VMH and LH. The different GLP-1R gene expression in response to glucose in certain hypothalamic

nuclei suggests that GLP-1R gene expression would not only be tissue-specific but also even cell-specific in defined brain areas. These findings also support the usefulness of hypothalamic slices for these kinds of studies because they express the opposite effects observed in vivo between VMH and LH.

Because the concentrations of extracellular glucose in the brain seem to be lower than those in blood (from 0.5 to 4.5 mmol/L) [33], we used a broad range of glucose concentrations from 0.5 to 20 mmol/L to get general information of the effects of glucose on GLP-1R expression. We did observe effects of glucose within this physiological range but also at 20 mmol/L glucose; this could be explained in terms of the presence of neurons that are stimulated by an increase from 5 to 20 mmol/L glucose [34]. Obviously, 20 mmol/L of glucose can be considered as a pharmacological concentration; but these levels may be found in the blood of uncontrolled diabetic patients, and the results obtained under this circumstance may be of interest from a pathophysiological point of view. We also found that regulatory peptides have specific effects depending on the tissue location. Thus, leptin stimulated in a dose- and time-dependent manner (data not shown) the GLP-1R mRNA levels in the VMH, as was the case with GT1-7 cells, whereas NPY did not change it. However, they had a different action in the LH, in apparent agreement with the well-known distinctive functions observed in this area for other parameters. Thus, NPY, which acts with antagonist function to leptin, decreased significantly the GLP-1R expression in LH.

The actions of GLP-1 on VMH and LH contribute to feeding termination and satiety [35-37]. Orexigenic and anorexigenic peptides located in the VMH, LH, paraventricular, and arcuate nuclei interact with one another in such a way that they can induce a characteristic feeding behavior. The cooperative effects between these 2 types of regulatory peptide can also be considered, as in the case of the stimulating action of GLP-1(7-36)amide on corticotropin-releasing hormone (CRH) neurons [38], which enhances the effects of both peptides on the inhibition of feeding. Furthermore, leptin reduces food intake and body weight by inhibiting the activity of hypothalamic neurons that produce orexigenic peptides, such as NPY, melanin-concentrating hormone, galanin, and agouti-related peptide [39-41]. Thus, leptin receptors are present on NPY neurons in the hypothalamus [42]; and the mutation of these receptors leads to the insensitivity of these animals to the inhibitory action of leptin on NPY expression [43]. According to our results, it seems to be possible that the anorexigenic action of leptin is reinforced by its effect on a greater expression of the GLP-1 receptor and favored by decreased levels of NPY. In addition, leptin stimulates the activity of neurons containing anorexigenic peptides, such as CRH, neurotensin, $\alpha$-melanocyte stimulating hormone, and cocaine- and amphetamine-regulated transcripts [39-41]. In fact, both the CRH receptor and the melanocortin-4 receptor antagonists are able to block the anorexigenic effects of leptin [44]. Furthermore, leptin increases the synthesis of GLP-1 in the nucleus of the tractus solitarius and the content of this peptide in hypothalamic nuclei [45], whereas exendin(9-39)—the specific antagonist of GLP-1—blocked both effects. These findings suggest that leptin inhibits food intake, at least in part, by activation of GLP-1 synthesis by specific neurons and, according to our results, by stimulating the expression of GLP-1 receptors.

Antagonist effects between GLP-1 and NPY as shown in this work by the blocking action of NPY on the stimulated effect of leptin on GLP-1 receptor expression are similar to the reduced neuronal activity of GLP-1 and leptin during fasting that favors the release of NPY from the arcuate nucleus, whereas the administration of leptin to fasted animals inhibits this increase in NPY and reduces food intake [46]. These findings can be explained in terms of the notion that NPY neurons that are activated by fasting would be the ones that express the long form [47] of the leptin receptor. It is noteworthy that where NPY neurons do not express significant amounts of the long form of the leptin receptor, they are not activated by fasting. In addition, anorexigenic peptides may also facilitate the shift of the fasting to the fed status [43,47] and hence play an important role in the adaptive response to negative energy balance. In summary, our findings support a distinctive response in VMH and LH hypothalamic areas to glucose oscillations in the extracellular space and likewise a different modulation by orexigenic and anorexigenic peptides in these areas. Thus, the stimulating effect of leptin on GLP-1R expression with no changes in NPY-induced activity could enhance the anorexic actions generated through this receptor.

## Acknowledgments

The authors are indebted to Dr Lankat-Buttgereit and Prof P Mellon for their generous gifts of the human GLP-1R promoter and GT1-7 cell line, respectively. We thank Dr L Frago for her valuable help in real-time PCR determinations. This work was supported by grants from the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Red de Diabetes y Enfermedades Metabólicas Asociadas REDIMET (RD 06/0015/0017), the Ministerio de Ciencia y Tecnología, and the Comunidad de Madrid, Spain. Dr Navas is supported by the Ramón y Cajal Program of the Spanish Ministry of Education and Science.

## References

[1] Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF. Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. J Biol Chem 1986;261: 11880-9.

[2] Kreymann B, Williams G, Ghatei MA, Bloom SR. Glucagon-like peptide-1 7-36: a physiological incretin in man. Lancet 1987;2:1300-4.

[3] Schjöldager BT, Mortensen PE, Christiansen J, Orskov C, Holst JJ. GLP-1 (glucagon-like peptide 1) and truncated GLP-1, fragments of human proglucagon, inhibit gastric acid secretion in humans. Dig Dis Sci 1989;34:703-8.
[4] Barragan JM, Rodriguez RE, Blazquez E. Changes in arterial blood pressure and heart rate induced by glucagon-like peptide-1-(7-36) amide in rats. Am J Physiol 1994;266:E459-66.
[5] Benito E, Blazquez E, Bosch MA. Glucagon-like peptide-1-(7-36) amide increases pulmonary surfactant secretion through a cyclic adenosine 3',5'-monophosphate–dependent protein kinase mechanism in rat type II pneumocytes. Endocrinology 1998;139:2363-8.
[6] Vara E, Arias-Diaz J, Garcia C, Balibrea JL, Blazquez E. Glucagon-like peptide-1(7-36) amide stimulates surfactant secretion in human type II pneumocytes. Am J Respir Crit Care Med 2001;163: 840-6.
[7] Thorens B. Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. Proc Natl Acad Sci U S A 1992;89:8641-5.
[8] Wei Y, Mojsov S. Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences. FEBS Lett 1995;358: 219-24.
[9] Alvarez E, Roncero I, Chowen JA, Thorens B, Blazquez E. Expression of the glucagon-like peptide-1 receptor gene in rat brain. J Neurochem 1996;66:920-7.
[10] Galehshahi FS, Goke B, Lankat-Buttgereit B. Contribution of a PS1-like element to the tissue- and cell-specific expression of the human GLP-1 receptor gene. FEBS Lett 1998;436:163-8.
[11] Wildhage I, Trusheim H, Goke B, Lankat-Buttgereit B. Gene expression of the human glucagon-like peptide-1 receptor is regulated by Sp1 and Sp3. Endocrinology 1999;140:624-31.
[12] Mora F, Exposito I, Sanz B, Blazquez E. Selective release of glutamine and glutamic acid produced by perfusion of GLP-1 (7-36) amide in the basal ganglia of the conscious rat. Brain Res Bull 1992;29:359-61.
[13] Turton MD, O'Shea D, Gunn I, et al. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 1996;379:69-72.
[14] Navarro M, Rodriquez de Fonseca F, Alvarez E, et al. 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 1996;67:1982-91.
[15] Alvarez E, Martinez MD, Roncero I, et al. 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 2005;92:798-806.
[16] Mizuno Y, Oomura Y. Glucose responding neurons in the nucleus tractus solitarius of the rat: in vitro study. Brain Res 1984; 307:109-16.
[17] Oomura Y, Yoshimatsu H. Neural network of glucose monitoring system. J Auton Nerv Syst 1984;10:359-72.
[18] Oomura Y, Ono T, Ooyama H, Wayner MJ. Glucose and osmosensitive neurones of the rat hypothalamus. Nature 1969;222:282-4.
[19] Ashford ML, Boden PR, Treherne JM. Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K+ channels. Pflugers Arch 1990;415:479-83.
[20] Dunn-Meynell AA, Routh VH, Kang L, Gaspers L, Levin BE. Glucokinase is the likely mediator of glucosensing in both glucose-excited and glucose-inhibited central neurons. Diabetes 2002;51: 2056-65.
[21] Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 1990;5:1-10.
[22] Lee K, Li B, Xi X, Suh Y, Martin RJ. Role of neuronal energy status in the regulation of adenosine 5'-monophosphate-activated protein kinase, orexigenic neuropeptides expression, and feeding behavior. Endocrinology 2005;146:3-10.
[23] Wetsel WC. Immortalized hypothalamic luteinizing hormone–releasing hormone (LHRH) neurons: a new tool for dissecting the molecular and cellular basis of LHRH physiology. Cell Mol Neurobiol 1995;15: 43-78.
[24] House SB, Thomas A, Kusano K, Gainer H. Stationary organotypic cultures of oxytocin and vasopressin magnocellular neurones from rat and mouse hypothalamus. J Neuroendocrinol 1998;10:849-61.
[25] Arima H, House SB, Gainer H, Aguilera G. Direct stimulation of arginine vasopressin gene transcription by cAMP in parvocellular neurons of the paraventricular nucleus in organotypic cultures. Endocrinology 2001;142:5027-30.
[26] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. New York: Academic Press; 1986.
[27] Sharp FR, Sagar SM, Swanson RA. Metabolic mapping with cellular resolution: c-fos vs. 2-deoxyglucose. Crit Rev Neurobiol 1993;7: 205-28.
[28] Chaudhuri A, Zangenehpour S, Rahbar-Dehgan F, Ye F. Molecular maps of neural activity and quiescence. Acta Neurobiol Exp (Wars) 2000;60:403-10.
[29] Sanz C, Roncero I, Vazquez P, Navas MA, Blazquez E. Effects of glucose and of insulin on glucokinase activity in rat hypothalamus. J Endocrinol 2007;193:259-67.
[30] Yang Y, Zhou L, Liu S, Tang J, Li F, Li R, et al. Expression of feeding-related peptide receptors mRNA in GT1-7 cell line and roles of leptin and orexins in control of GnRH secretion. Acta Pharmacol Sin 2005; 26:976-81.
[31] Beak SA, Heath MM, Small CJ, et al. Glucagon-like peptide-1 stimulates luteinizing hormone–releasing hormone secretion in a rodent hypothalamic neuronal cell line. J Clin Invest 1998;101: 1334-41.
[32] Solomon A, De Fanti BA, Martinez JA. Peripheral ghrelin participates in the glucostatic signaling mediated by the ventromedial and lateral hypothalamus neurons. Peptides 2006;27:1607-15.
[33] Silver IA, Erecinska M. 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 1994;14: 5068-76.
[34] Fioramonti X, Lorsignol A, Taupignon A, Penicaud L. A new ATP-sensitive K+ channel-independent mechanism is involved in glucose-excited neurons of mouse arcuate nucleus. Diabetes 2004;53: 2767-75.
[35] Larsen PJ, Tang-Christensen M, Jessop DS. Central administration of glucagon-like peptide-1 activates hypothalamic neuroendocrine neurons in the rat. Endocrinology 1997;138:4445-55.
[36] Schick RR, Zimmermann JP, vorm Walde T, Schusdziarra V. Peptides that regulate food intake: glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats. Am J Physiol Regul Integr Comp Physiol 2003;284:R1427-35.
[37] Acuna-Goycolea C, van den Pol A. Glucagon-like peptide 1 excites hypocretin/orexin neurons by direct and indirect mechanisms: implications for viscera-mediated arousal. J Neurosci 2004;24: 8141-52.
[38] Gotoh K, Fukagawa K, Fukagawa T, et al. Glucagon-like peptide-1, corticotropin-releasing hormone, and hypothalamic neuronal histamine interact in the leptin-signaling pathway to regulate feeding behavior. FASEB J 2005;19:1131-3.
[39] Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 1999;20:68-100.
[40] Hakansson ML, Brown H, Ghilardi N, Skoda RC, Meister B. Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 1998;18:559-72.
[41] Elmquist JK, Maratos-Flier E, Saper CB, Flier JS. Unraveling the central nervous system pathways underlying responses to leptin. Nat Neurosci 1998;1:445-50.

[42] Mercer JG, Hoggard N, Williams LM, et al. Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. J Neuroendocrinol 1996;8:733-5.

[43] Schwartz MW, Baskin DG, Bukowski TR, et al. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes 1996;45:531-5.

[44] Uehara Y, Shimizu H, Ohtani K, Sato N, Mori M. Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin. Diabetes 1998;47:890-3.

[45] Goldstone AP, Morgan I, Mercer JG, et al. Effect of leptin on hypothalamic GLP-1 peptide and brain-stem pre-proglucagon mRNA. Biochem Biophys Res Commun 2000;269:331-5.

[46] Baskin DG, Breininger JF, Schwartz MW. Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 1999;48:828-33.

[47] Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG. Identification of targets of leptin action in rat hypothalamus. J Clin Invest 1996;98:1101-6.