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Research article

# Regulation of the expression of LXR in rat hypothalamic and hippocampal explants

María Sol Kruse[a,*], Lucas Gabriel Suarez[a], Héctor Coirini[a,b]

[a] Laboratory of Neurobiology, Instituto de Biología y Medicina Experimental-CONICET, Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina
[b] Depto. de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, C1121ABG Buenos Aires, Argentina

## HIGHLIGHTS

- LXR in the hypothalamus is sensible to glucose, insulin and lipids levels in vitro.
- LXR in the hippocampus was affected only by glucose treatment in vitro.
- Insulin and glucose decrease LXRβ in the hypothalamus while not affecting LXRα.
- Colic acid and cholesterol increase LXRα in hypothalamus while not affecting LXRβ.
- Glucose increases both LXRα and LXRβ expression in the hippocampus in vitro.

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## ABSTRACT

Liver X receptors (LXR) are important transcription factors involved in the regulation of carbohydrate and lipid metabolism and are expressed in different brain areas. Recently we described that LXR expression in the hypothalamus is sensitive to serum levels of lipids and carbohydrates. Here, we further characterized the effects of glucose, insulin, cholesterol and cholic acid on the expression of LXRα and LXRβ in hypothalamus and hippocampus explants as in vitro models. The LXR activation products, GLUT2 and ABCA1, were also analyzed by Western blot. Glucose had different effects in the hypothalamus compared to the hippocampus. In the hypothalamus, increases in glucose concentrations decreased LXRβ expression while in the hippocampus increased both receptor subtypes levels. In contrast, insulin treatment decreased LXRβ in the hypothalamus while having no effects on the hippocampus. Cholic acid and cholesterol increased only LXRα expression in the hypothalamus whereas no effects on the hippocampus were detected. The newly expressed LXR receptors may be functional active since the level of the LXR activation product ABCA1 was also increased. Changes in GLUT2 expression was observed only when LXRβ levels were increased. Altogether these data show that LXR are sensitive to glucose, insulin and lipids in vitro, as well as in vivo as we previously showed, suggesting an involvement of LXR in central metabolic pathways and control of energy homeostasis.



## 1. Introduction

The prevalence of type 2 diabetes and Alzheimer's disease (AD) is steadily increasing in the developed world, as a result of increasing obesity and longevity of individuals. There is growing evidence suggesting a link between the two diseases. Both subtypes of Liver X receptors, LXRα and LXRβ, are nuclear receptors involved in the regulation of carbohydrate and lipid metabolism. LXR activation in the brain facilitates cellular cholesterol excretion, reduces the deposition of amyloid plaques and improves cognitive deficits [1,2]. Therefore LXR are being considered promising targets to treat diseases such as atherosclerosis and Alzheimer's. Despite the growing importance of LXR in the brain, little is known about their function and location in the CNS. In the hypothalamus, in the supraoptic (SOA) and paraventricular nuclei (PVN), LXRβ regulates arginine vasopressin (AVP) expression and is implicated in the control of water balance in both brain and kidney [3]. Furthermore, the expression of thyrotropin releasing hormone (TRH) and melanocortin receptor type 4 (MC4R) is repressed by activation of LXR in the hypothalamus [4]. Recent studies from our laboratory show that LXR receptors are expressed in different brain areas but

* Corresponding author at: Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina.
*E-mail address:* sol.kruse@conicet.gov.ar (M.S. Kruse).

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only in the hypothalamus is their expression sensitive to serum glucose, insulin and triglycerides levels. Specifically, hypothalamic LXR expression correlates negatively with serum insulin and triglyceride but only LXRβ correlates negatively with the area under the curve (AUC) produced during the glucose tolerance [5,6]. Moreover, altered levels of hypothalamic LXRβ and a change from negative to positive LXRβ-AUC correlation were found in two animal models with metabolic abnormalities [5,6]. These studies show a relationship between glucose and lipid homeostasis and the expression of LXR in the hypothalamus indicating that LXR in this brain area may have a regulatory function on the intermediate metabolism. Despite all of this, whether glucose, insulin or lipids could directly affect LXR expression is unknown. In this project we study the in vitro regulation of LXR in the hypothalamus by glucose, insulin, cholesterol and cholic acid. Cholic acid was tested as it has recently been identified as an endogenous LXR agonist [7]. In parallel, we analyzed the effects in the hippocampus for comparison to another SNC area that does not seem to be critical for LXR regulation of glucose. The expression of LXR activation products, ABCA1 and GLUT2, were also analyzed to further asses the functionality these receptors.

## 2. Materials and methods

### 2.1. Animals and ex vivo cultures

Animal procedures were approved by the Animal Care and Use Ethical Committee of the School of Medicine, University of Buenos Aires, in accordance with guidelines defined by the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals procedures. Animals were kept under standard laboratory conditions at 24 °C, with 12 h light:12 h darkness cycles and food and water ad libitum. Male Sprague–Dawley rats weighing 210 g (n = 6) were killed by decapitation and the hypothalamus and the hippocampus were rapidly dissected out. Explants of hypothalamus and hippocampus were used as in vitro models [8,9]. To aid the diffusion of the medium and ensure the exposure to metabolite treatments, tissues were cut approximately equal width on a chilled Petri dish and placed in oxygenated fresh aCSF solution for 30 min. Each hippocampus was divided transversely in 2–3 mm slices and placed in the same well. The hypothalami were cut longitudinally by the rostral-caudal medline, and both halves were placed in the same well [10]. The tissues were cultured in a six-well culture dish containing 1 ml of DMEM, 1% FBS, 10 μg/ml streptomycin and glutamine pH 7,4 at 37 °C/5% $CO_2$ [10], with the addition of different concentrations of glucose (5.5, 8.5, 15.5, 25.5 mM), insulin (2.5, 5, 10 nM), cholesterol (0.7, 1.6 mM), cholic acid (10, 50 μM) for 2, 4 and 6 h. Medium for control hemisections was diluted with vehicle. When incubation time was finished the explants were quickly frozen on dry ice and stored at −80 °C.

### 2.2. Solution preparations

The cholesterol solution was prepared by mixing 1 g of cholesterol in 10 ml of ethanol (250 mM). The solution was then heated gently, stirring until the cholesterol was dissolved. The final cholesterol concentration was measured in the incubation media by spectrophotometry (Wiener Labs S.A.I.C., Rosario, Argentina). Cholic acid was diluted in methanol and glucose and insulin were dissolved in sterile distilled water. In all cases, stock solutions were prepared 200× concentrated.

### 2.3. LDH measurement

Measurement of LDH activity in the extracellular medium was performed as a quantitative method for assessing cell injury by using a cytotoxicity kit assay (Cyto Tox 96 nonradiactive, Promega) [11]. Explants treated with $H_2O_2$ (7% v/v) for 2 h were used as positive control to determine the viability of the explants. $H_2O_2$ exposed explants presented greater LDH levels (approximately 650% more) compared to the LDH values obtained from the explants used for the study (0.005–0.012 mg/ml).

### 2.4. Western blotting

Homogenates were prepared by sonication in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM $Na_3VO_4$, and 1% Triton 100, pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics) as previously described [11,12]. A total of 20 mg of protein was separated by 10% SDS–PAGE in Tris–glycine electrophoresis buffer at 120 V for 90 min. Proteins from gels were transferred onto PVDF membranes (Bio-Rad), and the membranes were blocked with TBS-T (20 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; and 0.1% Tween-20) containing 5% fat-free milk for 1 h. Blocked membranes were incubated with the primary antibody in TBS-T containing 5% fat-free milk at 4 °C overnight. The primary antibodies used were LXRα (1:1000, Abcam, Cambridge, UK), LXRβ (1:1000, Abcam), ABCA1 (1/700, Abcam), GLUT2 (1/500, Abcam) and F-actin (1:1000, Santa Cruz Biotechnology) [5]. Immunoblots were then washed with TBS-T three times and incubated at room temperature for 1 h with the respective HRP-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, Buenos Aires, Argentina). Chemiluminescence was detected with the ECL system (GE Healthcare Life Sciences) and exposure to hyperfilm (GE Healthcare Life Sciences). All membranes were then stripped and reprobed for F-actin as a loading control. Signals in the immunoblots were scanned and analyzed by Scion Image Software (National Institutes of Health, Washington DC, USA). The amount of target protein was indexed to F-actin in all cases to ensure correction for the amount of total protein on the membrane. The results were reported as percentages of values obtained from expression of target proteins compared to controls.

### 2.5. Statistical analysis

Values are expressed as mean ± SEM. At least two similar but separate experiments were evaluated in all cases containing samples from three to four different animals per treatment. The significances among variables were evaluated using three-way ANOVA and/or two-way ANOVA and then one-way ANOVA followed by Fisher's post-hoc test or Student's *t*-test for two-group comparisons. In all cases, the Statview Statistical Software (SAS Institute, Inc., Cary, NC, USA; v5.0.1) was used. Differences were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Effect of glucose on LXRα and LXRβ expression in explants of rat hypothalamus and hippocampus

The effect of different glucose concentrations (5.5, 8.5, 15.5 and 25.5 mM) at different incubation times (2, 4 and 6 h) in hypothalamus and hippocampus explants were studied to examine whether changes in energy status in vitro, such as low or high levels of glucose, can affect expression of LXRα and LXRβ. As shown in Fig. 1B, increasing glucose concentration produced a decrease in LXRβ expression in the hypothalamus with respect to 5.5 mM at

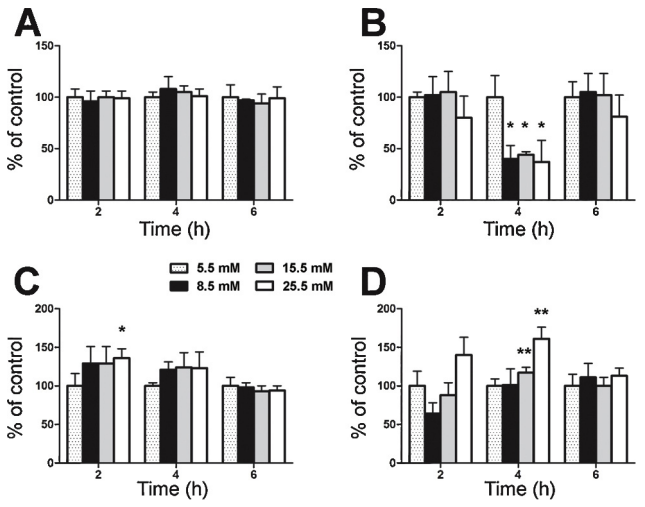


**Fig. 1.** LXRα (A, C) and LXRβ (B, D) expression in the hypothalamus (A, B) and the hippocampus (C, D) explants after 2, 4 and 6 h of treatment with glucose 5.5, 8.5, 15.5, 25.5 mM. Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Data are presented as mean ± S.E.M and significant differences between treatments were identified by one-way ANOVA followed by Fisher's post-hoc test. *p < 0.05 and **p < 0.01.

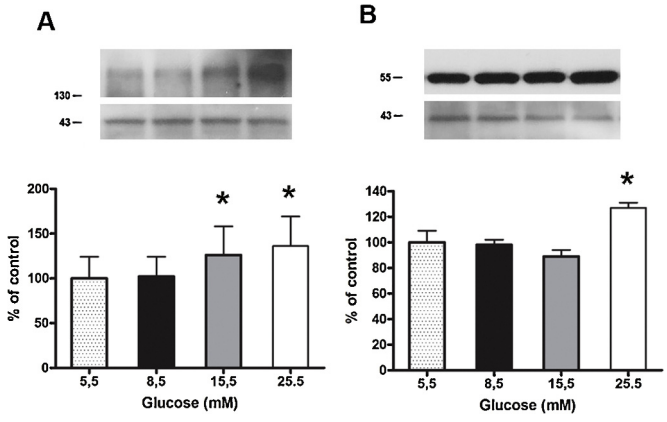


**Fig. 2.** ABCA1 (A) and GLUT2 (B) expression in the hippocampus explants after 4 h of treatment with glucose 5.5, 8.5, 15.5 or 25.5 mM. Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of ABCA1, GLUT2 and F-actin expression are shown in the upper panel. Data are presented as mean ± S.E.M and significant differences between treatments were identified by one-way ANOVA followed by Fisher's post-hoc test. *p < 0.05.

4 h (60%, 56% and 63%, for glucose 8.5, 15.5 and 25.5 mM respectively, p < 0.05) while there were no differences on LXRα expression (Fig. 1A).

In the hippocampus explants an increase in LXRβ (4 h, 17% and 61%, 15.5 and 25.5 mM of glucose respectively, p < 0.01) (Fig. 1D) and LXRα (2 h, 36%, glucose 25.5 mM, p < 0.05) was found (Fig. 1C). To assess whether these newly LXR were functional active, the expression of the LXR activation products, ABCA1 and GLUT2, were measured. Both ABCA1 (4 h, 26% and 35%, 15.5 and 25.5 mM of glucose respectively, p < 0.05) and GLUT2 expression (4 h, 27%, glucose 25.5 mM, p < 0.05) were increased in the hippocampus (Fig. 2). No changes in ABCA1 or GLUT2 were observed in the hypothalamus.

### 3.2. Effect of insulin on LXRα and LXRβ expression in explants of rat hypothalamus and hippocampus

The effect of different concentrations of insulin (2.5, 5 and 10 nM) in the presence of 5.5, 8.5 or 25.5 mM glucose at different incubation times (4 and 6 h) on the expression of LXRα and LXRβ in vitro was also evaluated. Insulin treatment affected LXRβ expression in the hypothalamus explants only in the presence of 8.5 mM glucose at 4 h. In the presence of 8.5 mM of glucose, insulin produced a decrease of LXRβ expression (4 h; insulin 2.5 nM: 27%; insulin 5 nM: 40%; insulin 10 nM: 56%; p < 0.01) (Fig. 3B). LXRα was not affected by insulin in this tissue. In the hippocampus no changes were observed by insulin treatment

in any of the conditions tested (4 and 6 h, insulin: 2.5, 5 or 10 nM, in the presence of glucose 5.5, 8.5 or 25.5 mM) (Fig. 3A and C).

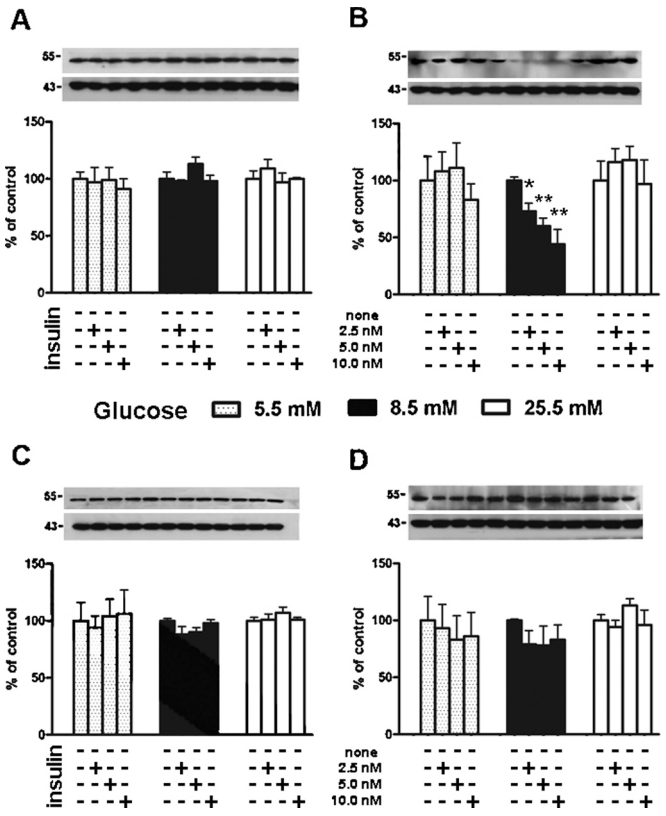


**Fig. 3.** Western blot of LXRα (A, C) and LXRβ (B, D) in the hypothalamus (A, B) and the hippocampus (C, D) explants. Cultures were treated with insulin 2.5, 5 or 10 nM for 4 h in the presence of glucose 5.5, 8.5 or 25.5 mM. Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of LXR and F-actin expression are shown in the upper panel. Data are presented as mean ± S.E.M and significant differences between treatments were identified by one-way ANOVA followed by Fisher's post-hoc test. *p < 0.05 and **p < 0.01.

### 3.3. Effect of cholic acid and cholesterol on LXRα and LXRβ expression in explants of rat hypothalamus and hippocampus

In order to evaluate the effect of cholic acid (CA) (10 and 50 μM) and cholesterol (chol) (0.7 and 1.6 mM) at different times (4 and 6 h) explants from both tissues were incubated in vitro. LXRα expression increased in the hypothalamus after 4 h of treatment with CA or chol (CA 10 μM: 23%, CA 50 μM: 19%, chol 0.7 mM: 17% and chol 1.6 mM: 21%, p < 0.05) (Fig. 4A). ABCA1 expression, but not GLUT2, was increased by CA treatment (CA 10 μM: 36%, CA 50 μM: 64%, p < 0.05) (Fig. 5). No changes were found in LXRβ expression by the different treatments in any tissue (Fig. 4B and D). No differences were observed after 6 h of treatment (data not shown).

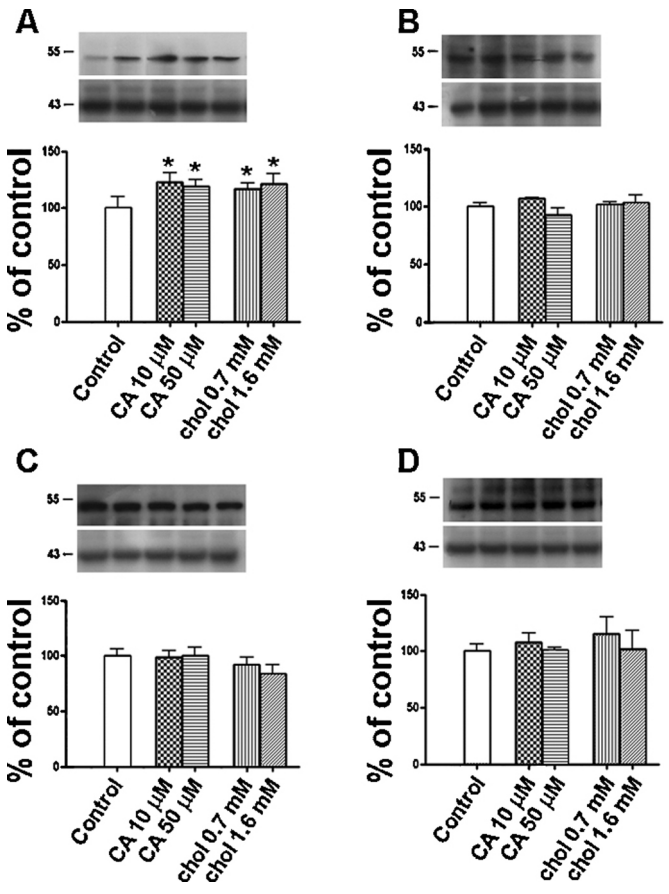


**Fig. 4.** Western blot of LXRα (A, C) and LXRβ (B, D) in the hypothalamus (A, B) and the hippocampus (C, D) explants. Cultures were treated with CA (10 or 50 μM) or chol (0.7 or 1.6 mM) for 4 h. Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of LXR and F-actin expression are shown in the upper panel. Data are presented as mean ± S.E.M and significant differences between treatments were identified by one-way ANOVA followed by Fisher's post-hoc test. *$p < 0.05$.

## 4. Discussion

In this study we found that glucose treatment in vitro decreases LXRβ expression in the hypothalamus. These results are in accordance with our previous studies in vivo as the area under the curve (AUC) during the glucose tolerance test correlates negatively with the levels of hypothalamic LXRβ [5,6]. In addition, LXRβ expression is decreased in the hypothalamus of two animal models of glucose intolerance [5,6]. Notably, LXRα was unaffected by in vitro glucose treatment in the hypothalamus. In vivo, only intolerant animals induced by high fructose diet exhibit increased levels of LXRα in the hypothalamus [5]. However, there is no correlation between AUC and LXRα indicating that hypothalamic LXRβ are more sensitive to glucose changes than LXRα [5].

In the hippocampus glucose increased both LXRβ and LXRα in vitro. These newly identified LXR receptors are probably functional, since the expression of the LXR activation products, GLUT2 and ABCA1, were also increased. In our in vivo models we previously found no long-term effects of hyperglycemia on LXR in the hippocampus. However, the levels of LXRα and LXRβ are increased at one day of age in the hippocampus of male rats born to diabetic dams [6] and the glycemia of these animals at this age is still high [13]. Thus, the glucose regulation of LXR expression differs in the hippocampus compared to the hypothalamus in vivo and in vitro, indicating that glucose probably activates different intracellular pathways in each brain area. In fact in neurons of the hippocampus, glucose, through GLUT2, is supposed to regulate synaptic activity and neurotransmitter release whereas in hypothalamic neurons, GLUT2 serves as a glucose sensor in regulation of food intake [14]. In hypothalamic explants glucose regulates the expression of the orexigenic factors neuropeptide Y and Agouti-related protein through the phosphorylation states of AMP-activated protein kinase (AMPK) [8]. It would be interesting to test whether glucose also regulates the expression of LXR via AMPK.

D-Glucose and D-glucose-6-phosphate are endogenous LXR agonists in vitro, with efficacy comparable to that of oxysterols [15]. More recently, increased glucose levels were found to increase LXRα abundance and its product sterol regulatory element-binding protein type 1c (SREBP1c) or to decrease the fructose 1,6-bisphosphatase (FBPase) in the hypothalamus of rainbow trout in vitro and in vivo [16,17]. Altogether this demonstrates the

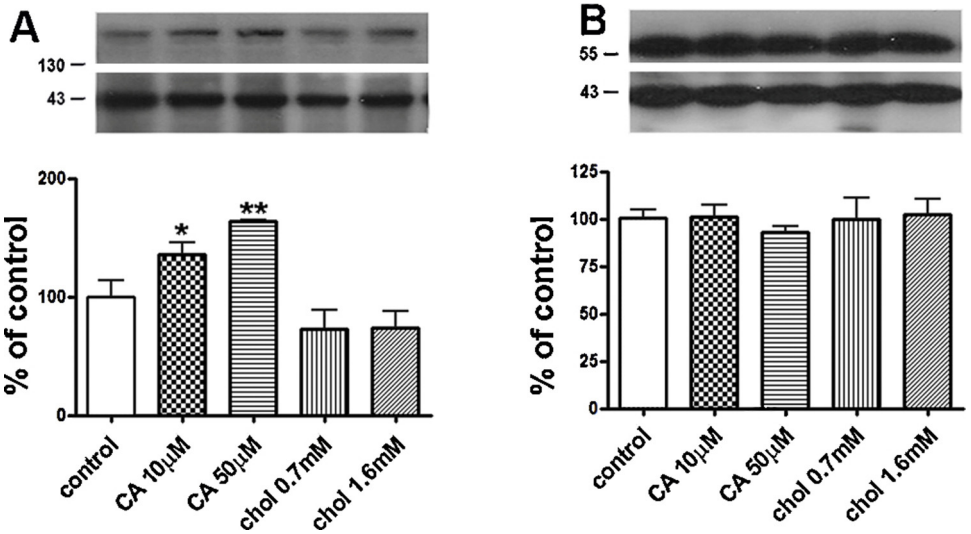


**Fig 5.** ABCA1 (A) and GLUT2 (B) expression in the hypothalamus explants after 4 h of treatment with CA (10 or 50 μM) and chol (0.7 or 1.6 mM). Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of ABCA1, GLUT2 and F-actin expression are shown in the upper panel. Data are presented as mean ± S.E.M and significant differences between treatments were identified by one-way ANOVA followed by Fisher's post-hoc test. *$p < 0.05$ and **$p < 0.01$.

presence of a functioning LXR-based glucosensing system in the hypothalamus. Further studies are necessary to characterize the underlying mechanisms and the physiological role of this putative LXR dependent-glucosensing system in the hypothalamus.

Insulin treatment in vitro decreased LXRβ expression in the hypothalamus only in the presence of 8.5 mM glucose. The LXRβ decrease could be due to an increase of glucose bioavailability in the neurons as the hypothalamus is the only brain region that expresses the insulin-sensitive glucose transporter GLUT-4. Therefore, the hypothalamus, but not the hippocampus, may take up glucose in response to insulin [18] and in turn, decrease the LXRβ. In contrast, insulin did not affect LXRα expression in any of the studied conditions. While insulin increases the expression of LXRα in the liver by promoting the stability of the transcript [19], this does not seem to be the case in the hypothalamus or the hippocampus. Insulin did not affect the expression of LXRα in vitro and serum insulin correlates negatively only with hypothalamic LXR in vivo [5].

The abundance of insulin receptors (IR) is higher in the hypothalamus compared to the hippocampus [20–22]. Interestingly, in the hypothalamus IR match the LXR localization as they are expressed in the arcuate (ARC), SOA, PVN nuclei and are less dense in the ventromedial nucleus (VMN) [22]. In contrast, IR in the hippocampus are found in pyramidal cells [23] and so far, no one has described the presence of LXR in this neuronal type. Altogether this data suggests that insulin could directly affect LXR in the hypothalamus, but not in the hippocampus.

In this study we found similarities between our in vitro and in vivo models, however, it is not surprising to have also found some discrepancies. The intolerant animal models we previously used presented hypertriglyceridemia and hyperinsulinemia [5,24] among other disturbances [25,26]. Therefore, there are other factors in the animal models that could be affecting LXR expression in a direct or indirect way. In the in vitro model, we subscribe only to the hypothalamus/hippocampus bypassing the effects of others metabolites and hormones produced in the periphery. Nevertheless, here we showed that glucose, insulin and cholesterol treatment affect LXR expression in vitro in an isoform specific way, showing that at least some of the effects observed in vivo are most likely mediated by these pathways.

In contrast to what was observed with glucose and insulin, the treatment with the lipids exerted different effects in vitro. Cholic acid and cholesterol increased LXRα expression in the hypothalamus after 4 h treatment. Cholic acid treatment also increased the expression of ABCA1 but not GLUT2. The increased ABCA1 indicates that these newly LXRα are functional active. It also suggests that LXRβ, but not LXRα, are responsible for GLUT2 activation. Indeed, GLUT2 expression was shown to be increased by LXR activation only in pancreatic beta-cells and INS-1E insulinoma cells which express only the LXRβ isoform [27].

LXRα (but not LXRβ) has been shown to be controlled by an autoregulatory mechanism. Activation of LXR upregulates LXRα in murine and human macrophages [28], myocytes [29], and adipocytes [30]. This autoregulatory capacity could favor the induction of LXRα over LXRβ [30]. In accordance with these results we found that cholic acid, which has recently been identified as an endogenous ligand of LXR [7], promotes the expression of LXRα over LXRβ at least in the hypothalamus. Even though cholesterol increased LXRα expression, this may not be produced by a direct activation on LXR as cholesterol treatment failed to increase ABCA1. The hypothalamus does not express CYP46A1 the enzyme responsible for the formation of 24-S-hydroxicholesterol from cholesterol, an endogenous LXR agonist and the most abundant oxysterol in the brain [31]. Cholesterol may be then acting through a signaling pathway. A recent proteomics study identified a large number of cholesterol-binding proteins (i.e., >250), suggesting a potential link between cholesterol and diverse cellular processes yet to be determined [32].

Altogether these results suggest that LXRβ are mainly sensitive to carbohydrate changes while LXRα respond to lipid changes in the hypothalamus. The importance of LXRα and LXRβ on lipid and carbohydrate homeostasis is particularly evident in the knockout models. The phenotype of transgenic mice deficient of LXRα notes the importance of this receptor in lipid metabolism. These animals have high cholesterol and are unable to handle dietary cholesterol overload which determine greater hypercholesterolemia and massive hepatic accumulation of this lipid [33]. They also show a reduction in the content of bile acids and a resistance to stimuli that normally promote their synthesis, indicating a defect in the final catabolism of cholesterol. On the other hand, transgenic mice deficient of LXRβ demonstrate the relevance of this receptor in the metabolism of carbohydrates. These animals show glucose intolerance due to a marked reduction in pancreatic insulin secretion and a loss of sensitivity to insulin for glucose uptake [34].

In the hypothalamus LXR are located in different nuclei [5]. LXRα is expressed in the PVN and the VMN nuclei while LXRβ is found in the ARC. Both isoforms are present in the medial preoptic area (mPOA). In addition, LXR expression was found in the nuclei of magnocellular neurons [3] and in TRH-parvocellular neurons of the PVN [4]. On this basis we could speculate that the lipid effects on LXRα are more relevant in the mPOA, PVN and VMN hypothalamic areas, while the glucose-LXRβ effects are in the mPOA, PVN and ARC. These areas are interconnected and they control a variety of physiological responses including feeding, metabolism, fertility and cardiovascular regulation, among others. It will be important to identify the neuronal populations involved in the LXR-regulation by carbohydrates and lipids to understand the physiological relevance of this modulation in the hypothalamus.

In summary, in this study we show that LXR expression is regulated by glucose, insulin and lipids, in accordance to our previous studies in vivo, suggesting that LXR are involved in the central control of the intermediate metabolism. In line with these results, it has recently been shown that expression of MC4R in the hypothalamus is under regulation of LXR [4]. Moreover, MC4R in the hypothalamus regulates the hepatic cholesterol metabolism as it facilitates hepatic cholesterol synthesis and HDL cholesterol transport [35]. These last two pathways are also under control of LXR in the liver [36]. Altogether these results strongly support the hypothesis that hypothalamic LXR play a role in the central control of the intermediate metabolism and energy homeostasis.

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