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Research article

# LXR activation increases the expression of GnRH AND αMSH in the rat hypothalamus *in vivo*

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ABSTRACT

Liver X receptors (LXR) are important transcription factors involved in the regulation of carbohydrate and lipid metabolism. Recently, we described LXR receptors expression in the hypothalamus but their function in this brain area remains unknown. Here, we evaluated the function of LXR on the expression of factors produced in the hypothalamus *in vitro* and *in vivo* by Western blotting and immunocytochemistry. More precisely we studied the expression of GnRH and GHRH, αMSH and NPY in male Sprague-Dawley rats. The effects of two synthetic LXR agonists, T0901317 and GW3965, were first tested *in vitro*. Hypothalamic explants were treated with either T0901317 or GW3965 (10 μM) for 2, 4, 6 and 8 h. As a positive control the cholesterol ABCA1 and glucose GLUT2 transporters were used. No changes were observed in the expression of the factors evaluated *in vitro*.

The effects of the LXR agonists were then tested *in vivo*. Rats were injected ICV into the third ventricle with either T0901317 or GW3965 (2.5 μg/5 μL ICV) and after 3.5 h or 24 h the hypothalami were dissected out and rapidly frozen for analysis. αMSH and GnRH expression was significantly increased after 3.5 h of T0901317 treatment. Anterior/posterior hypothalamic ratio increases for αMSH expression and decreases for GnRH expression after 24 h of LXR activation. Altogether these results show that LXR activation affects the expression of GnRH and αMSH, suggesting that LXR in the hypothalamus is capable of modulating hypothalamic responses related to appetite, sexual behavior and reproductive functions.

## 1. Introduction

Liver X receptors (LXR), LXRα and LXRβ, are nuclear receptors involved in the regulation of carbohydrate and lipid metabolism that are now emerging as new drug targets. In the brain LXR activation facilitates cellular cholesterol excretion, reduces the deposition of amyloid plaques and improves cognitive deficits [1,2]. Therefore LXR are being considered 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 and paraventricular nuclei, LXRβ regulates arginine vasopressin (AVP) expression and it is implicated in the control of water balance in both brain and kidney [3]. The expression of thyrotropin releasing hormone (TRH) and melanocortin receptor type 4 (MC4R) by thyroid hormone (TH) is repressed by activation of LXR in the hypothalamus [4,5]. Recent studies from our laboratory show that LXR are expressed in different brain areas but only the expression of these receptors in the hypothalamus is sensitive to serum glucose, insulin and triglycerides [6,7] indicating a link between hypothalamic LXR and the intermediate metabolism. In the hypothalamus we found LXRα expressed in the paraventricular (PVN) and the ventromedial (VMN) nuclei while LXRβ are present in the arcuate nucleus (ARC). Both isoforms are expressed in the medial preoptic area (mPOA). Moreover, altered levels of hypothalamic LXR were found in two animal models with metabolic abnormalities [6,7]. The presence of glucosensing mechanisms dependent on LXR in the hypothalamus has also been described [8–10]. Altogether these studies show a close relationship between the carbohydrate and lipid homeostasis and the expression of LXR in the hypothalamus. However, whether LXR could trigger neuronal responses related to the control of food intake and energy expenditure is still unknown. In this project we examined the role of LXR in the hypothalamus through the characterization of some of the neuropeptides regulated by LXR and its activation products *in vitro* and *in vivo*.

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## 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. 7-week-old Sprague–Dawley rats (n = 6) were killed by decapitation and the hypothalami were rapidly dissected out and placed in oxygenated fresh aCSF solution for 30 min. Explants of hypothalamus were used as *in vitro* model as described elsewhere [11,12]. Hypothalami were separated by transverse section to increase exposure to metabolites treatments. The tissues were then 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% $CO_2$ [8,13], with the addition of T0901317 (10 μM) or GW3965 (10 μM) for 2, 4, 6 and 8 h. Control hemisections were incubated with vehicle for the same times. When incubation time was finished the explants were quickly frozen on dry ice and stored at −80 °C.

### 2.2. LDH measurement

Measurement of the enzyme lactate dehydrogenase (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) [14]. 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).

### 2.3. ICV drug injection

9 to 10-week-old Sprague–Dawley male rats were anesthetized by ketamine-xylazine (80:4 mg/Kg Holliday/Richmond, Argentina) and placed in a stereotaxic apparatus. A 10 μL-Hamilton syringe was placed in the 3 V, 2.3 mm posterior to Bregma, on the midline, and 8.5 mm ventral to the brain surface of the rat brain atlas of Paxinos and Watson (1998). 5 μL (1 μL per minute) solution containing 2.5 mg of GW3965 or T0901317 was injected in aCSF with methylene blue (1 mg/mL) as indicator of the injected zone. After 5 min the needle was slowly withdrawn and animals were removed from the stereotaxic apparatus and sutured. Tramadol (0.05%) was offered to the animals in the drinking water as a painkiller. Control animals were injected ICV with vehicle alone (< 1% DMSO in aCSF + 1 mg/mL of blue methylene). After 3.5 h or 24 h one set of the animals were sacrificed by decapitation and hypothalami were rapidly dissected out and frozen on dry ice. Another set of animals were deeply anesthetized and perfused for immunocytochemical analysis.

### 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 [14,15]. 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 GHRH (1:1000, Abcam), GnRH (1:500, kindly provided by Dr. Damasia Becu, IByME-CONICET) [16,17], αMSH (1:1000, SIGMA) (García-Tornadú 2009), NPY (1:2000, Peninsula Laboratories) [18], ABCA1 (1/700, Abcam), GLUT2 (1/500, Abcam) and F-actin (1:1000, Santa Cruz Biotechnology) [7]. 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. Immunocytochemistry and fluorescent microscopy

The animals were deeply anesthetized by i.p. injection with chloral hydrate 28% (w/v, 0.1 mL/100 g of body weight) and the animals were fixed by intracardiac perfusion using 600 mL of 4% cold paraformaldehyde (PFH) in PBS, pH 7.4 [15]. The brains were removed immediately and left in 4% PFH overnight. They were then washed with PBS and the hypothalamus was sectioned with a vibratome. Coronal sections (70 mm thick; Bregma K0.26 to K3.20 mm) were collected and incubated in PBS containing 0.1% Tween 20 and 7% normal donkey serum for 1 h at room temperature. The tissue samples were then incubated with a rabbit anti-GnRH (1:500) and mouse anti-αMSH (1:1000, SIGMA) in PBS containing 2% donkey serum and 0.1% Tween 20 overnight at 4 °C. Subsequently, they were rinsed in PBS for 30 min and then incubated with donkey anti-mouse Cy3 and donkey anti-rabbit Cy3 (Millipore, all 1:300) for 2 h at RT. Nuclei were counterstained with DAPI (1 μg/mL). Finally, after washing, sections were mounted on glass slides and examined with a Olympus IX81-DSU Spinning Disk Confocal Microscope. All pictures were obtained under the same conditions; exposition time, lamp intensity and filters were kept constant throughout the experiment. The primary antibody was omitted in some sections as control; those were further processed under the same protocol described earlier. The fluorescence staining intensity from those sections was used as a marker to identify positive staining.

### 2.6. Quantification of the immunoreactive area

The immunoreactive area in each image was measured using the Scion Image Software (National Institutes of Health, Washington DC, USA). The Cy3 red staining was first filtered to prevent parameter overestimation. The immunopositive area was calculated as the ratio between the positive area and the corresponding subfield area, using a user-defined threshold as it was performed elsewhere [19].

### 2.7. 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 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 $p < 0.05$.

**Table 1**
LXR binding sites identified in the rat GnRH (1 and 2), GHRH (3 and 4) and POMC (5) promoter regions. Matrix family and individual matrix are the corresponding names of the sequences. Start/end position, starting/ending position of the consensus binding site in the sequence (relative to GnRH, GHRH or POMC); matrix similarity; matrix (groups of functionally similar transcription factors) similarity factor (0–1).

| | Matrix family | Individual Matrix | Start position | End position | Strand | Matrix similarity | Sequence |
|---|---|---|---|---|---|---|---|
| 1 | V$RXRF | V$LXRE.02 | 682 | 706 | (−) | 0.826 | ggaaaGTTCaaggataatggacgtc |
| 2 | V$RXRF | V$LXRE.02 | 409 | 433 | (−) | 1 | ctaaaGGTCaatgtccaacactgct |
| 3 | V$RXRF | V$LXRE.02 | 637 | 661 | (−) | 1 | tttcaGGTCaccagtcacatccctg |
| 4 | V$RXRF | V$LXRE.02 | 302 | 326 | (−) | 0.783 | cctcgGCTCagagctccgcgcagac |
| 5 | V$RXRF | V$VDR_RXR.06 | 923 | 947 | (−) | 0.759 | gaagcgctgccaggaaGGTCacgtc |

## 3. Results

### 3.1. Effects of the LXR agonists T0901317 and GW3965 on the expression of the hypothalamic neuropeptides GnRH, GHRH, NPY, αMSH in vitro

Upon ligand induced activation, LXR bind to LXR response elements (LXRE) in the promoter regions of the target genes and regulate gene expression. LXRE consists of two idealized hexanucleotide sequences (AGGTCA) separated by four bases (DR-4 element) [20]. Using the database of genomic sequences MatInspector software version 8.1, Matrix Library 9.1 from the Genomatix suite v3.4 [21] we first screened for LXRE in numerous hypothalamic factors. We found that both GnRH and GHRH possess two LXRE sequences in their promoter region with matrix similarities close to 1 (maximum 1.00) (Table 1) and therefore they were considered as possible candidates to be regulated by LXR. We also examined whether the expression of αMSH is under LXR regulation in the hypothalamus as it has been previously established to be regulated by LXR in the pituitary [22] and MatInspector also identified a V$RXRF binding site which possess a LXRE1 sequence in the rat POMC promoter region (Table 1). The orexigenic neuropeptide, NPY was also studied as it is the counterpart of αMSH and it does not possess LXRE.

We first investigated whether LXR activation could affect neuropeptide gene expression *ex vivo*. Fresh hypothalami were cultured *ex vivo* and two synthetic LXR agonists, T0901317 and GW3965 (10 μM) were tested at different incubation times (2, 4, 6 and 8 h). Cultures were then homogenized in lysis buffer and subjected to Western blot. No significant changes were observed by T0901317 or GW3965 treatments, indicating that acute LXR activation does not affect the expression of any of the studied factors *in vitro* (Fig. 1A–D). Cultures were functional, active and responsive to LXR treatment as LXR activation increased the expression of the cholesterol transporter ABCA1 while not affecting the GLUT2 expression (Fig. 2A and B). Neuronal survival was also estimated by measuring LDH released in the incubation media. No changes in LDH incubation medium were found between control and treated tissues but they were highly increased in cultures exposed to $H_2O_2$ for 2 h (7% v/v, Fig. 2C).

### 3.2. Effects of a single ICV injection of LXR agonist on the expression of the hypothalamic neuropeptides GnRH, GHRH, NPY, αMSH

The effects of the two LXR agonists T0901317 and GW3965 were studied *in vivo* in adult male Sprague Dawley rats. A single dose of T0901317 or GW3965 was injected in the third ventricle using stereotaxic coordinates. As control, rats were injected with 5 μL of vehicle. After 3.5 h or 24 h of treatment animals were sacrificed and hypothalmi rapidly dissected out for Western blot analysis.

After 3.5 h the expression of GnRH and αMSH was increased by T0901317 treatment (34% and 12%, respectively, $p < 0.05$. Fig. 3B and D) while GW3965 produced a non-significant increase of GnRH expression. No changes were observed on the expression of GHRH or NPY (Fig. 3A and C). These results were supported by immunocytochemistry. Hypothalamic nuclei were identified by DAPI staining and the rat brain atlas of Paxinos and Watson (Paxinos G, Watson C 1998) (Fig. 4C and D). GnRH staining was densely punctate on a diffuse background in proximity to the perikarya (Fig. 4A and B). GnRH immunosignal was increased by T0901317 and GW3965 treatment in the mPOA (Fig. 4A) and ARC (Fig. 4B) but not in the VMN or the dorsomedial (DMH) nuclei of the hypothalamus (data not shown). αMSH labelling was also increased by LXR activation but only in the ARC by T0901317 treatment (Fig. 5). In the PVN, lateral hypothalamic area (LHA) or mPOA nuclei, αMSH immunosignal was not affected by T0901317 or GW3965 treatment (data not shown). The immunosignal was quantified and the GnRH and aMSH immunoreactive areas were measured in the mPOA and ARC hypothalamic nuclei. Only the T0901317 treatment significantly increased GnRH immunoreactive area in the mPOA (24.5%, $p < 0.05$) and in the ARC (14% $p < 0.05$). In addition, αMSH immunocreactive area was also significantly increased only by T0901317 treatment in the ARC (45.8% $p < 0.01$).

As we observed differences in the neuropeptide expression among the different hypothalamic nuclei by immunocitochemistry, we then divided the hypothalamus in two regions for the 24 h studies. The anterior hypothalamus (ATH) containing the mPOA, LHA and PVN was separated from the posterior hypothalamus (PHT) containing the ARC, VMN and DMH, by transversally cutting just behind the optic chiasm.

After 24 h of T0901317 ICV injection, GnRH expression decreased in the AHT by 43% ($p < 0.05$), whereas in the PHT increased by 135% ($p < 0.001$) (Fig. 6A). Thus, the AHT/PHT ratio decreased by LXR activation (control: 3.1 *vs.* treated: 0.5; $p < 0.001$) (Fig. 6A inset). In contrast, there was a non-significant increase in the expression of αMSH in the AHT while there was a decrease by 36% ($p < 0.05$) in the PHT (Fig. 6B) after 24 h of T0901317 ICV injection. The AHT/PHT ratio switched from 0.9 (control) to 1.8 (treated) ($p < 0.02$, Fig. 6B inset). No significant changes in the expression of NPY or GHRH were observed (data not shown).

## 4. Discussion

In this study we found that LXR activation in the hypothalamus increases the expression of GnRH and αMSH *in vivo*. To our knowledge, this is the first study describing that LXR is capable of regulating these neuropeptides in the hypothalamus. After 3.5 h of a single ICV dose of T0901317, GnRH expression increases both in the mPOA and the ARC nucleus while αMSH expression rises in the ARC. ATH/PHT ratio increases for αMSH expression and decreases for GnRH expression after 24 h of a single ICV T0901317 dose. These results suggest that acute LXR activation stimulates the GnRH/αMSH production whereas at longer times there is a release of these neuropeptides at distal areas from their production, most probably through the axon network. GnRH neurons are located in the septal-preoptic region and they project to the median eminence. In addition to hypophysiotropic axons projections, GnRH fibers also innervate the posterior hypothalamus and from there, GnRH neurons regulate distal areas such the ventral tegmental area and the midbrain central regions [23,24]. Thus, the increased GnRH expression observed at 24 h in the posterior hypothalamus may belong to this neuronal pathway.

The pulsatile release of GnRH into the hypophysial portal circulation [25,26] drives the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in anterior pituitary [27].

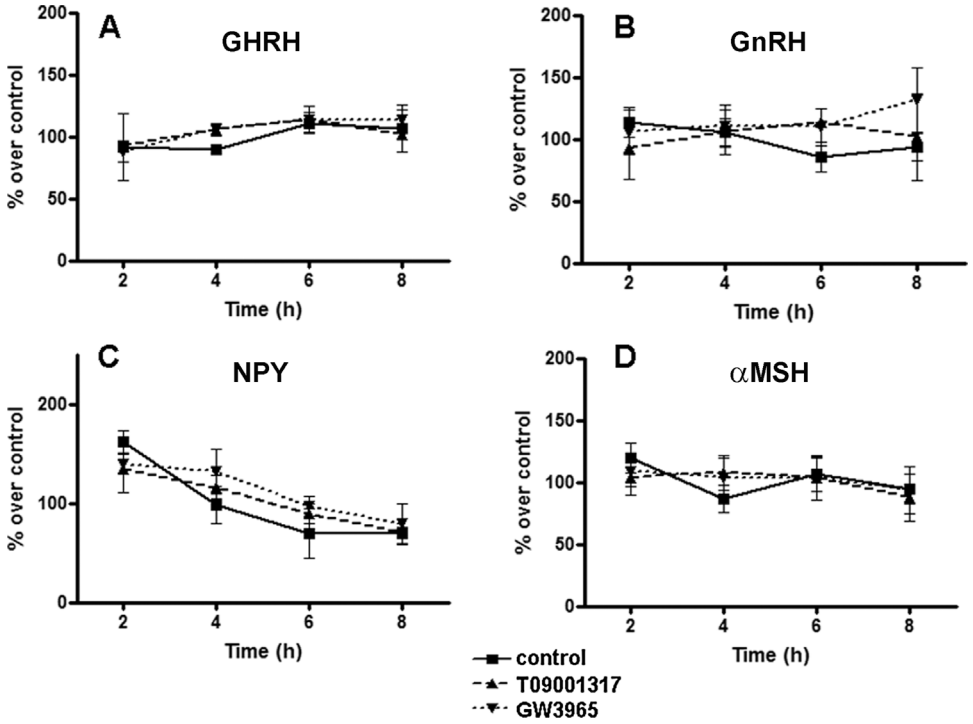


**Fig. 1.** Effect of LXR activation on the expression of different hypothalamic neuropeptides *in vitro*. *Ex vivo* cultures were treated with either T0901317 (triangle up) or GW3965 (triangle down) for 2, 4, 6 and 8 h. The expression of GHRH (A), GnRH (B), NPY (C) and αMSH (D) were analyzed by Western blot. Data are presented as mean ± S.D. from at least three independent experiments.

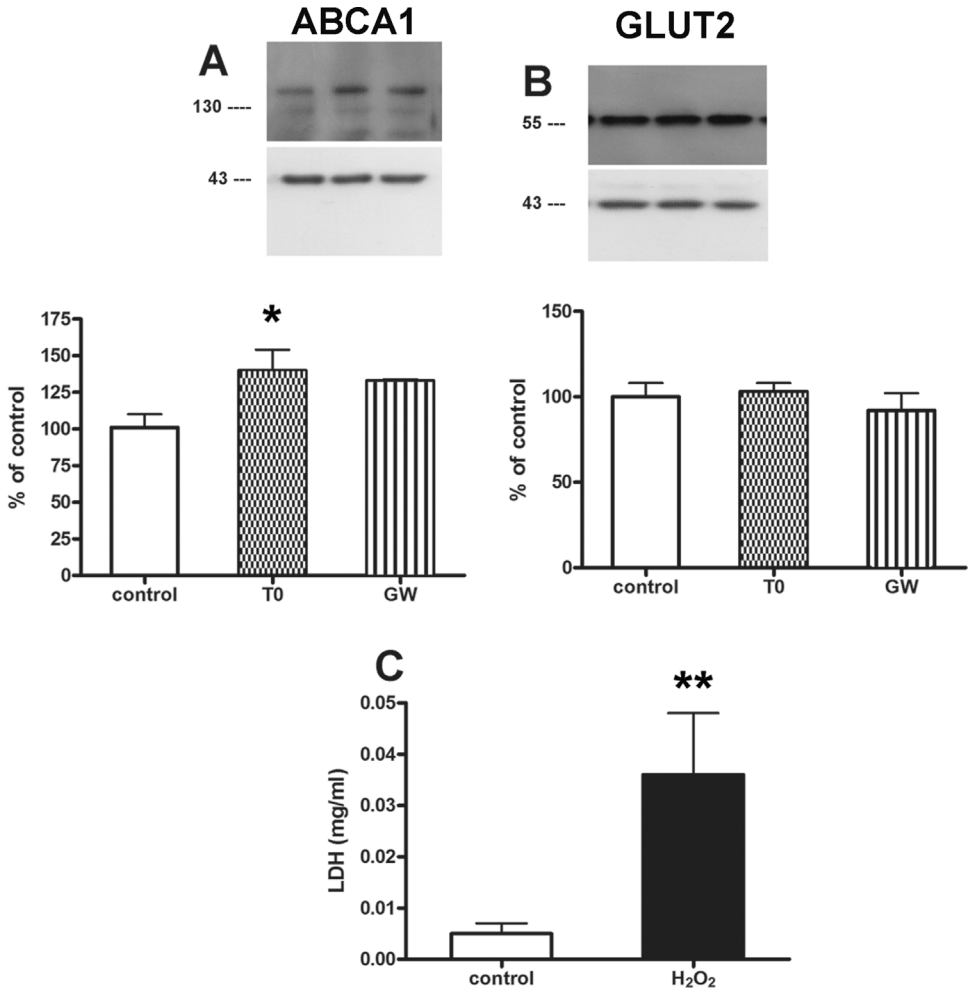


**Fig. 2.** The viability of the cultures was analyzed. Effect of GW3965 (GW) or T0901317 (T0) treatment on the expression of ABCA1 (A) and GLUT2 (B). Representative pictures of ABCA1, GLUT2 and the loading control F-actin are shown in the upper panel. C) LDH activity was measured in the incubation medium of the hypothalamic explants. Data are presented as mean ± S.D. from at least three independent experiments. *p < 0.05 and **p < 0.005, n = 7–10.

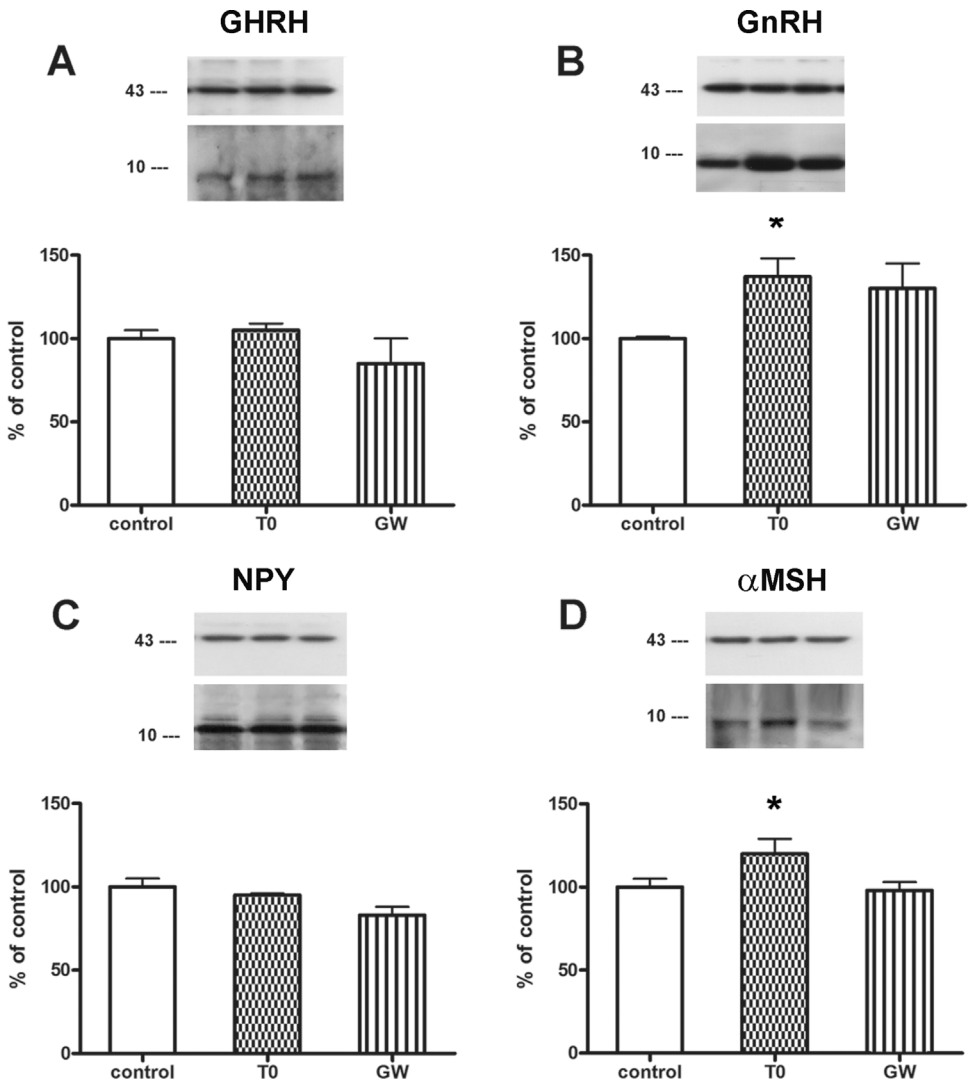


**Fig. 3.** Acute effect of a single ICV dose of T0901317 (T0) (patterned bars) or GW3965 (GW) (striped bars) on the expression of different hypothalamic neuropeptides. The expression of GHRH (A), GnRH (B), NPY (C) and αMSH (D) was determined by Western blot. Representative pictures of the neuropeptides and the loading control F-actin are shown in the upper panel. Data are presented as mean ± S.D. from at least three independent experiments. *p < 0.05, n = 6–8 animals/group.

In turn, circulating FSH and LH stimulate gametogenesis and the synthesis and secretion of testosterone in the testes and 17β-estradiol (E2) and progesterone in the ovaries. The importance of LXR on reproduction is increasingly being studied. Fertility is highly compromised in mice lacking LXRα, LXRβ or both genes (LXRα(-/-), LXRβ(-/-) and LXRαβ(-/-). Females have lower levels of progesterone, conceive less frequently and have significantly fewer pups [28,29]. LXRαβ(-/-) males become sterile when aging, showing an epididymal phenotype associated with dyslipidemia [30,31]. LXRα(-/-) male mice have lower levels of testicular testosterone and higher apoptotic rate of the germ cells while LXRβ(-/-) mice show increased lipid accumulation in the Sertoli cells and a lower proliferation rate of the germ cells [32,33]. Altogether, these data demonstrate that LXR play an important role in reproductive functions and collaborates to maintain both integrity and functions of the gonads, through the modulation at different levels of the GnRH-testosterone/progesterone axe. It would be interesting to study whether LXR could also affect LH/FSH production at the anterior pituitary level.

POMC in the hypothalamus, the αMSH precursor, is expressed in the ARC nucleus. Here we found that acute activation of LXR increases αMSH expression in the ARC while at longer times, αMSH levels increase in the anterior hypothalamus. In accordance with these results, LXR were shown to positively regulate the POMC gene promoter at the transcriptional level in the mouse and rat pituitary. T0901317 treatment increases POMC mRNA levels, the number of ACTH neurons and plasma ACTH and serum corticosterone levels *in vivo* [22]. In this study we have not observed any effects of LXR *in vitro*, suggesting that the effects are not directly mediated by LXR but through an intermediate or a co-activator. Nevertheless, we cannot discard that LXR could directly regulate the transcription of GnRH or POMC in the hypothalamus as they possess LXRE in their promoter region (Table 1) and it has previously been shown that LXR directly regulates the POMC transcription in the pituitary [22]. The exposition time to the drugs was different *in vitro* than *in vivo* (*i.e.* 2, 4, 6 and 8 h *vs.* one single ICV dose) and therefore, the bioavailability of the drug also differed between assays. Even though *ex vivo* cultures maintain some of the connections and structures present *in vivo*, in contrast to primary cultures or immortalized cell lines, the *in vivo* model seems to be more suitable to study the effects of LXR activation in the hypothalamus. Noteworthy, cultures were physiologically active and responded to LXR treatment as the LXR product, ABCA1 increased by LXR activation.

The biological effects of αMSH are largely mediated through melanocortin (MC) receptors but especially through the MC4R, αMSH is implicated in appetitive and obesity [34]. Hypothalamic MC4R is involved in the control of hepatic cholesterol metabolism by facilitating hepatic cholesterol synthesis and cholesterol transport [35], a pathway that is well-known to be regulated by liver LXR. Interestingly, it has recently been published that hypothalamic LXR activation regulates the TH-dependent transcriptional activity of MC4R [4]. In line with these studies we have previously shown that hypothalamic LXR expression is

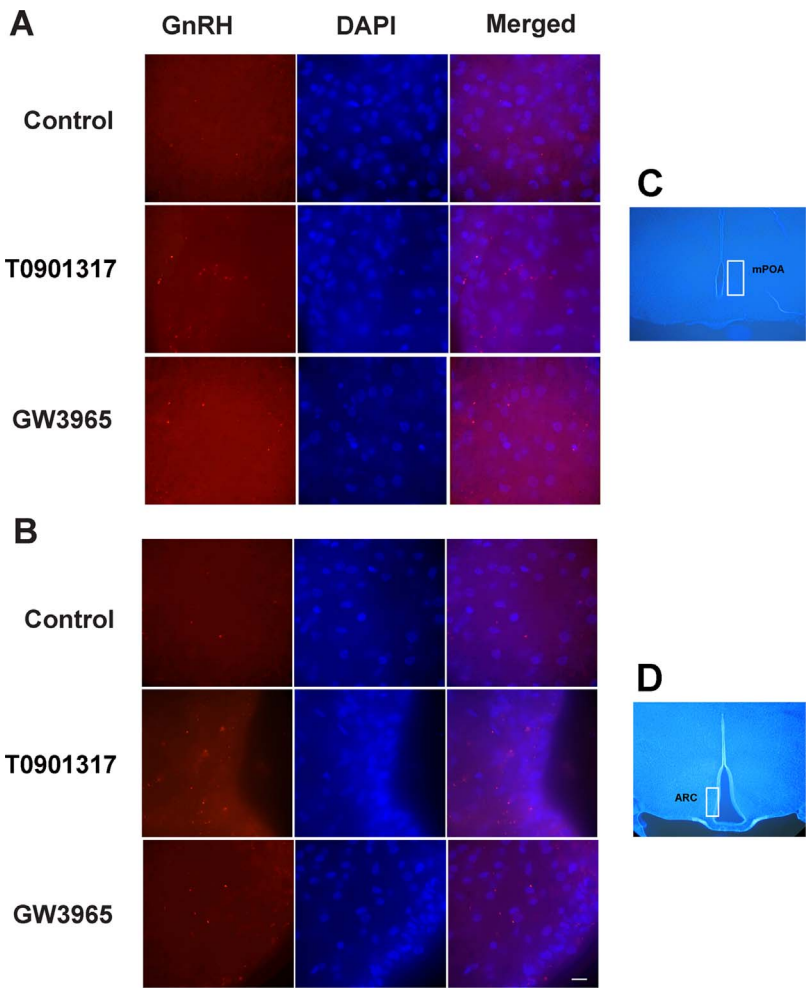


**Fig. 4.** Representative confocal microscopy images showing the immunoreactivity for GnRH in different hypothalamic nuclei: (A) mPOA, (B) ARC nucleus from control animals (upper row) and T0901317 (middle row) and GW3965 (lower row) treated animals. Data were obtained from 4 independent assays (n = 6–8). GnRH was labeled with Alexa Fluor 546 red fluorescence (first column). The second column shows nuclei stained with DAPI. The third column is the merged of both signals. Bar size = 25 μm.
(C and D) Low-magnification pictures indicating the region of each hypothalamic nuclei magnified in A and B respectively, identified by DAPI staining.

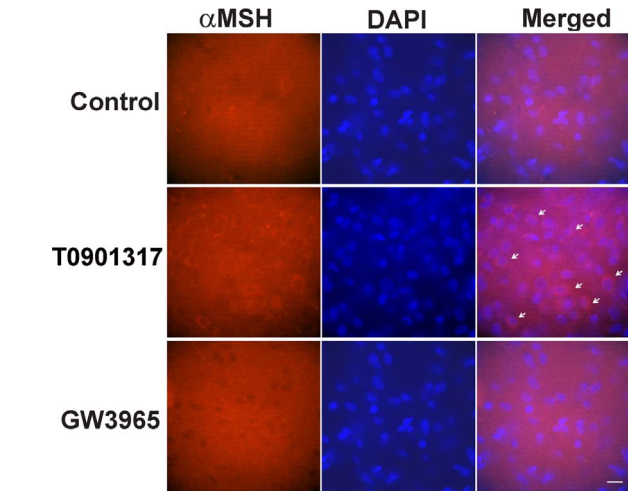


**Fig. 5.** Representative confocal microscopy images showing the immunoreactivity for αMSH in ARC nucleus from control animals (upper row) and T0901317 (middle row) and GW3965 (lower row) treated animals. Data were obtained from 4 independent assays (n = 6-8). αMSH was labeled with Alexa Fluor 546 red fluorescence (first column). The second column shows nuclei stained with DAPI. The third column is the merged of both signals. Bar size = 25 μm.

sensible to serum glucose, insulin and triglycerides [6,7] suggesting that LXR are involved in neuronal responses related to the control of food intake and energy expenditure.

Interestingly, the GnRH II, a variant of GnRH, was also shown to influence feeding behavior, as ICV administration of GnRH II induces a decrease in food intake [36–38] through the interaction with the αMSH-MC4R signalling pathway [39].

In summary, the present study shows that hypothalamic LXR is probably implicated in the central control of reproduction and energy balance by regulating some of their key proteins (i.e αMSH and GnRH). In accordance, previous studies from our laboratory demonstrate that LXR expression is altered in the hypothalamus of rats with metabolic abnormalities related to diabetes type II and metabolic syndrome [6,7]. Altogether the present study shows a possible link between hypothalamic LXR and the intermediate metabolism by interacting with, at least, the POMC-αMSH-melanocortin pathway involved in central metabolic regulation and the control of energy homeostasis.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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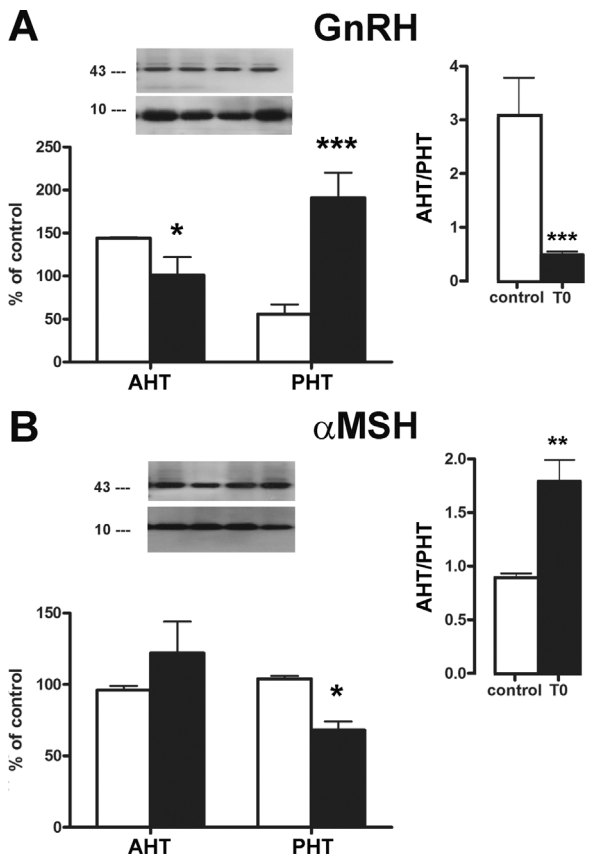


**Fig. 6.** Long-term effect of a single ICV dose of T0901317 (T0) (filled bars) on the expression of GnRH (A) and αMSH (B) in the anterior (AHT) and posterior hypothalamus (PHT). Representative pictures of GnRH, αMSH and the loading control F-actin are shown in the upper panel. The insets show AHT/AHP ratio of control *vs.* T0901317 treated animals. Data are presented as mean ± S.D. from at least three independent experiments. *p < 0.05, **p < 0.02, ***p < 0.001, n = 6–8 animals/group.

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