

# Alterations of LXR $\alpha$ and LXR $\beta$ expression in the hypothalamus of glucose-intolerant rats

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## Abstract

Liver X receptor (LXR)  $\alpha$  and  $\beta$  are nuclear receptors that are crucial for the regulation of carbohydrate and lipid metabolism. Activation of LXRs in the brain facilitates cholesterol clearance and improves cognitive deficits, thus they are considered as promising drug targets to treat diseases such as atherosclerosis and Alzheimer's disease. Nevertheless, little is known about the function and localization of LXRs in the brain. Here, we studied the expression of LXR in the brains of rats that received free access to 10% (w/v) fructose group (FG) in their beverages or water control drinks (control group (CG)). After 6 weeks rats in the FG presented with hypertriglyceridemia, hyperinsulinemia, and became glucose intolerant, suggesting a progression toward type 2 diabetes. We found that hypothalamic LXR expression was altered in fructose-fed rats. Rats in the FG presented with a decrease in LXR $\beta$  levels while showing an increase in LXR $\alpha$

expression in the hypothalamus but not in the hippocampus, cerebellum, or neocortex. Moreover, both LXR $\alpha$  and  $\beta$  expression correlated negatively with insulin and triglyceride levels. Interestingly, LXR $\beta$  showed a negative correlation with the area under the curve during the glucose tolerance test in the CG and a positive correlation in the FG. Immunocytochemistry revealed that the paraventricular and ventromedial nuclei express mainly LXR $\alpha$  whereas the arcuate nucleus expresses LXR $\beta$ . Both LXR immunosignals were found in the median preoptic area. This is the first study showing a relationship between glucose and lipid homeostasis and the expression of LXRs in the hypothalamus, suggesting that LXRs may trigger neurochemical and neurophysiological responses for the control of food intake and energy expenditure through these receptors.

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## Introduction

Liver X receptor (LXR)  $\alpha$  and  $\beta$  are ligand-activated transcription factors that belong to the nuclear receptor superfamily. Both LXRs are key sensors of intracellular sterol levels that trigger various adaptive mechanisms in response to cholesterol overload. These mechanisms include stimulation of reverse cholesterol transport and biliary cholesterol excretion, inhibition of intestinal absorption of dietary cholesterol, and suppression of cholesterol synthesis *de novo* (Baranowski 2008). LXRs are also involved in glucose homeostasis. It was recently demonstrated that LXR expression is increased in pancreatic  $\beta$  cells in type 2 diabetes (Choe *et al.* 2007). LXR stimulation normalizes glycemia and improves insulin sensitivity in rodent models of type 2 diabetes and insulin resistance (Cao *et al.* 2003, Laffitte *et al.* 2003,

Commerford *et al.* 2007) while not affecting glycemia in nondiabetic animals (Cao *et al.* 2003, Laffitte *et al.* 2003). Thus, in recent years, LXRs have emerged as promising targets to treat diseases such as atherosclerosis and type 2 diabetes (Luoma 2011).

The importance of the energy control of homeostasis by the CNS is now recognized. The CNS, especially through the hypothalamus, responds to adiposity, nutrient, and satiety signals by connecting neuroendocrine and autonomic pathways to regulate energy homeostasis and body weight (Bantubungi *et al.* 2011). LXRs are expressed in the CNS, although LXR $\beta$  is especially expressed at high levels (Schmidt *et al.* 1999, Whitney *et al.* 2002). Nevertheless, the distribution of LXR expressions in the brain and their physiological function, in particular with respect to brain control of energy homeostasis, remains to be clarified.

As LXRs are implicated in various metabolic processes, we evaluated the expression of LXRs in control rat CNS and compared it with a rat model of insulin resistance and glucose intolerance (Sleder *et al.* 1980, Zavaroni *et al.* 1980). We show that the expression of both LXR $\alpha$  and  $\beta$  are altered in the hypothalamus of glucose-intolerant animals with hypertriglyceridemia and hyperinsulinemia, suggesting a role of hypothalamic LXRs in lipid and glucidic homeostasis.

Materials and Methods

Experimental animals

Sprague Dawley rats weighting 250–300 g were used in this study. Animal procedures have been approved by the Animal Care and Ethical Use Committee of the School of Medicine, University of Buenos Aires, Argentina, in accordance with guidelines defined by the European Communities Council Directive (86/609/EEC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals procedures. Animals were maintained on a 12 h light:12 h darkness cycle with food available *ad libitum*. Over 6-week period, all animals were given unrestricted access to tap water (control group (CG)) or a 10% fructose solution (w/v made up on tap water, fructose group (FG)). All efforts were made to reduce the number of animals used and to minimize suffering.

Glucose tolerance test

After 6 weeks, food and fructose drink were removed and the animals fasted for 10 h. Blood samples were taken from the tail vein of each animal and fasting glucose levels were determined using a commercial strip and a glucometer (OneTouch Ultra, Johnson & Johnson, CABA, Argentina). A glucose load was administered by i.p. injection (2 g/kg body weight) and blood glucose levels were measured at, 30, 60, and 120 min post-injection. The area under the glucose curve (AUC) during the glucose tolerance test was calculated using the trapezoidal method of integration. All animals were subsequently returned to their cages and given their original test diet for further 24 h and then fasted again for 10 h prior killing.

Insulin and lipid blood levels

Rats were killed by decapitation; blood samples were collected from trunk blood and immediately centrifuged for 5 min at room temperature at 1800 *g* in a tabletop centrifuge. The serum was collected and triglycerides, total cholesterol, LDL, and HDL were measured by spectrophotometry (Wiener Labs S.A.I.C., Rosario, Argentina). Insulin was determined by a solid-phase chemiluminescent enzyme immunoassay (Acris Antibodies, Santiago de Chile, Chile).

Western blotting

After decapitation, hypothalamus, hippocampus, cerebellum, neocortex, and liver were rapidly dissected out and stored at –80 °C (Coirini *et al.* 1983). Homogenates were prepared by sonication in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1 mM PMSE, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1% Triton 100, pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics) as described previously (Kruse *et al.* 2009a,b). Protein (20  $\mu$ g) was separated on 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 membranes were blocked with TBS–T (20 mmol/l Tris, pH 7.5; 150 mmol/l NaCl, and 0.1% Tween-20) containing 5% of 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 LXR $\alpha$  (1:1000, Abcam, CABA, Argentina), LXR $\beta$  (1:1000, Abcam) (Morales *et al.* 2008, Stayrook *et al.* 2008, Tian *et al.* 2009), and F-Actin (1:1000, Santa Cruz Biotechnology, CABA, Argentina). Immunoblots were then washed with TBS–T three times and incubated at RT for 1 h with the respective HRP-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, CABA, Argentina). Chemiluminescence was detected with the ECL system (GE Healthcare Life Sciences) and exposed to hyperfilm (GE Healthcare Life Sciences). Signals in the immunoblots were scanned and analyzed by Scion Image Software.

Immunocytochemistry and confocal 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

**Table 1** Body weight and fasting plasma levels of glucose and insulin at the end of the 6-week diet. The 2-h blood glucose level during the glucose tolerance test is also shown in the table. Data are expressed as mean  $\pm$  s.d. from at least three independent experiments

Experimental group	Body weight (g)	Glycemia (mg/dl)	Insulin ( $\mu$ U/ml)	2-h Blood glucose levels (mg/dl)
CG	495 $\pm$ 51	78.25 $\pm$ 6.17	22.22 $\pm$ 12.79	121.13 $\pm$ 20.3
FG	496 $\pm$ 48	85.50 $\pm$ 5.60*	37.21 $\pm$ 12.86*	153.80 $\pm$ 20.6†

\**P* < 0.05, †*P* < 0.01 compared with CG (*n* = 12 per group).

**Table 2** Fasting plasma levels of triglycerides, cholesterol, LDL, and HDL at the end of 6-week diet. Data are expressed as mean  $\pm$  s.d. from at least three independent experiments

Experimental group	Triglycerides (g/l)	Cholesterol (g/l)	LDL (g/l)	HDL (g/l)
CG	1.18 $\pm$ 0.41	0.83 $\pm$ 0.64	0.58 $\pm$ 0.52	0.29 $\pm$ 0.11
FG	1.44 $\pm$ 0.78*	1.02 $\pm$ 0.70	0.75 $\pm$ 0.58	0.31 $\pm$ 0.12

\* $P < 0.05$  compared with CG ( $n = 12$  per group).

the animals were fixed by intracardiac perfusion using 600 ml of 4% cold paraformaldehyde (PFH) in PBS, pH 7.4. The brains were removed immediately and left in 4% PFH overnight. Then they were washed with PBS and the hypothalamus was sectioned with a vibratome. Coronal sections (70  $\mu$ m thick; Bregma  $-0.26$  to  $-3.20$  mm) were collected and incubated in PBS containing 0.1% Tween 20 and 3% normal goat serum for 1 h at room temperature. The tissue samples were then incubated with a rabbit anti-LXR $\alpha$  receptor (1:300, Abcam) and mouse anti-LXR $\beta$  receptor (1:300, Abcam) in PBS containing 2% goat serum and 0.1% Tween 20 overnight at 4 °C. Subsequently, they were rinsed in PBS for 30 min and then incubated with goat anti-mouse 488 and goat anti-rabbit 546 (Immunochim, CABA, Argentina, all 1:200) for 2 h at RT. Nuclei were counterstained with DAPI. Finally, after washing, sections were mounted on glass slides and examined with a Leica TCS SP inverted confocal scanning laser microscope. The primary antibody was omitted in some sections as control; those were processed under the same protocol described earlier. The fluorescence staining intensity from those sections was used as a marker to identify positive staining.

### Statistical analysis

Values are expressed as mean  $\pm$  s.d. At least three similar but separate experiments were evaluated in all cases containing samples from three to four different animals per treatment. Results were evaluated using Student's *t*-test for two-group comparison or one-way ANOVA. The correlations were also analyzed by ANOVA. In all cases, the Statview Statistical Software (SAS Institute, Inc., Cary, NC, USA; v5.0.1) was used. Differences were considered significant at  $P < 0.05$ .

## Results

### Body weight, basal lipid levels, and glucose tolerance test

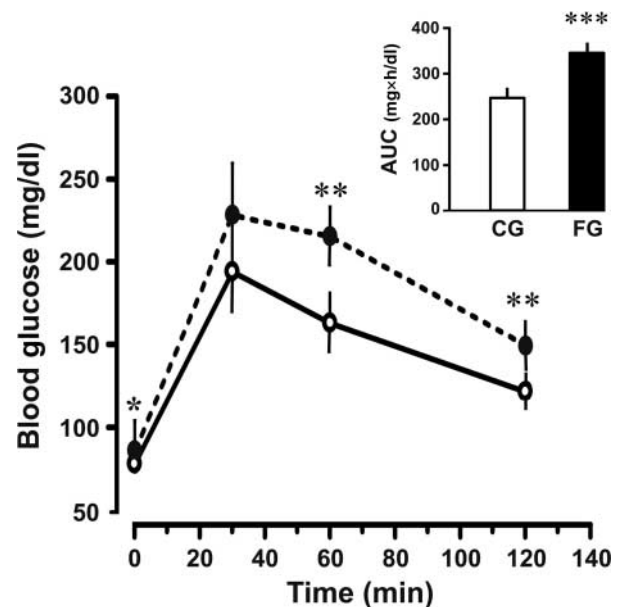
After 6 weeks of fructose treatment, the animals' body weights showed nonsignificant differences between groups, as shown in Table 1. Fasting blood glucose and insulin (INS) levels were higher in rats given fructose drinks than controls (Table 1, FG vs CG). On the other hand, FG animals had significantly higher levels of fasting triglycerides (TAG) than controls

but no significant differences were found in cholesterol, LDL, and HDL levels (Table 2).

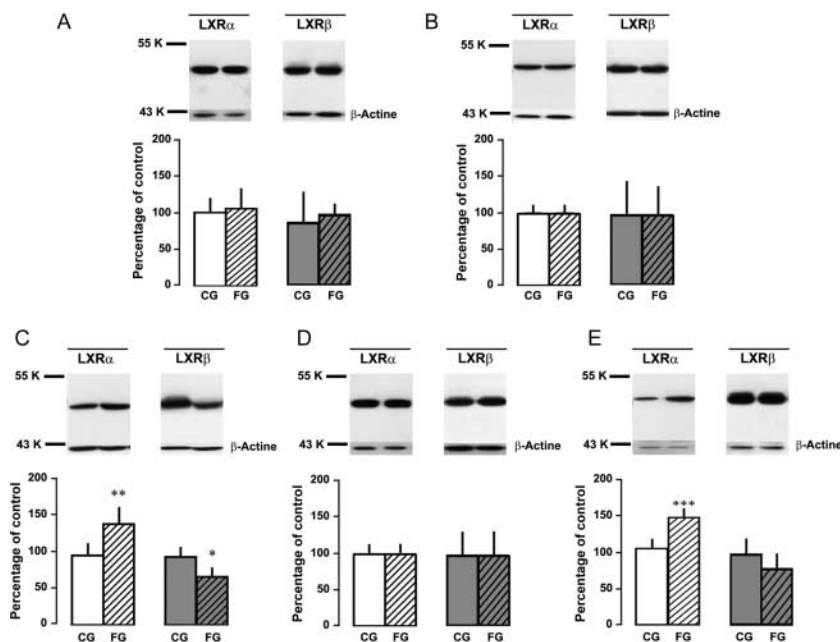
Animals' ability to regulate a glucose load was determined after i.p. injection of glucose solution (2 g/kg). FG rats displayed glucose intolerance, showing significant changes at 60 and 120 min (Fig. 1 and Table 1). A significant difference between CG and FG in the AUC during a glucose tolerance test was also observed (Fig. 1 inset).

### LXR $\alpha$ and $\beta$ expression in different brain areas

LXR expressions were assessed by western blot analysis in neocortex, hippocampus, hypothalamus, cerebellum, and liver. Both receptors were expressed differently in animals given fructose drinks compared with the water controls. LXR $\alpha$  was increased by 43% in FG compared with CG in the



**Figure 1** Blood glucose levels during an intraperitoneal glucose tolerance test performed 6 weeks after initiation of fructose treatment in FG (filled circle) and CG rats (open circle). Values are expressed as the mean  $\pm$  s.d. from 12 animals from each group (\*\* $P < 0.01$  FG vs CG). Inset: values of the areas under the blood glucose curves during the 2-h tolerance test (AUC) in CG and FG obtained by trapezoidal integration. Data are expressed as the mean  $\pm$  s.d. from at least three independent experiments (\*\*\*)  $P < 0.0005$ .



**Figure 2** Western blot of LXR $\alpha$  (white bars) and LXR $\beta$  (gray bars) in the CG (empty bars) and FG (striped bars). Data were quantified by densitometric analysis and corrected for the F-actin loading control. (A) Neocortex. (B) Hippocampus. (C) Hypothalamus. (D) Cerebellum. (E) Liver. Representative pictures of LXR expression and F-actin loading control are shown above each bar. Data are presented as mean  $\pm$  s.d. from at least three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0005$  compared with CG,  $n = 7$ –10 animals/group.

hypothalamus (Fig. 2C, white bars) but not in the neocortex, hippocampus, or cerebellum (Fig. 2A, B and D respectively, white bars). In accordance with previous studies (Tobin *et al.* 2002), liver LXR $\alpha$  was significantly increased in FG (64%; Fig. 2E, white bars). On the other hand, LXR $\beta$  was found significantly attenuated in the hypothalamus of FG compared with CG (26%; Fig. 2C, gray bars).

No differences were detected for LXR $\beta$  expression in other brain regions studied (Fig. 2A, B and D, gray bars). Because LXR expression was significantly changed in the hypothalamus, we evaluated the localization of LXRs in the hypothalamic nuclei by immunocytochemistry followed by confocal microscopy analysis. LXR $\alpha$  signal was observed in the paraventricular (PVN) and ventromedial (VMN) nuclei while LXR $\beta$  signal was found in the arcuate (ARC) nucleus (Fig. 3). In addition, both LXR immunosignals were detected in the median preoptic area (mPOA) often expressed in different cell types (Fig. 3). LXR $\alpha$  immunosignal appeared to be increased and LXR $\beta$  immunosignal was attenuated in the FG compared with the CG (Fig. 3, first and second columns respectively).

#### Correlation between LXRs and insulin, triglycerides, or AUC

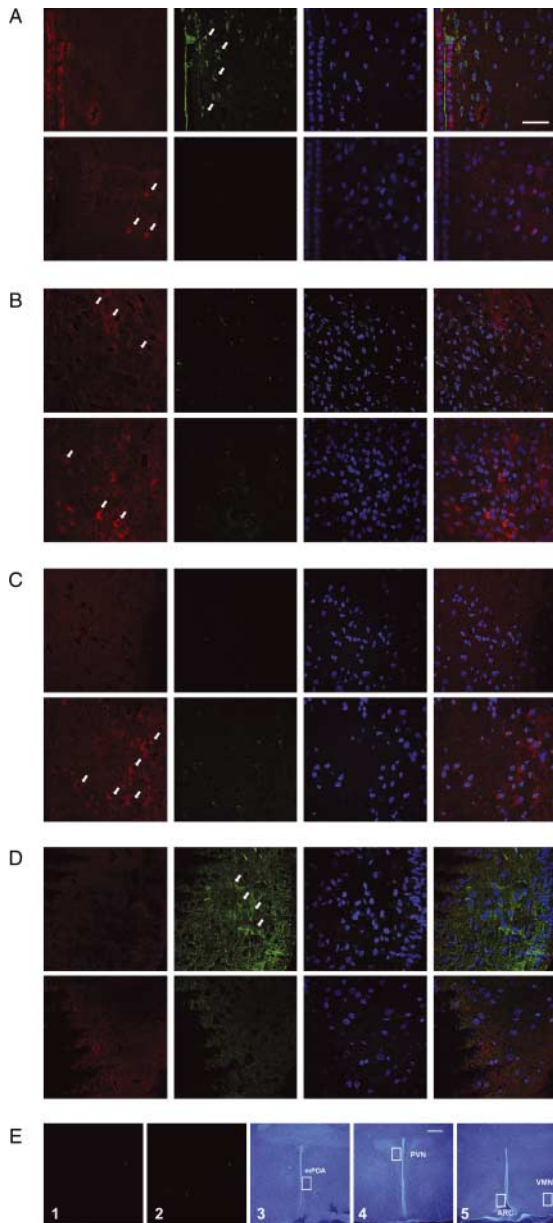
LXRs are involved in the regulation of lipid and glucose metabolism. On the other hand, the hypothalamus plays a crucial role in the regulation of food intake and energy expenditure (Schwartz *et al.* 2000, Dowell *et al.* 2005).

Because significant alterations in LXRs were found in the hypothalamic region, the expression of these receptors with the circulating levels of INS and TAG were compared. In addition, the relationship between LXR expression and AUC values was also evaluated.

Statistical analysis of INS and TAG levels for each individual animal showed a significant correlation with the expression of the hypothalamic LXRs (Fig. 4). Similar statistical parameters were found for INS or TAG and LXR $\alpha$  when all animals were considered (INS:  $F(1;15) = 5.54$ ,  $P = 0.033$ ;  $r^2 = 0.27$ ; TAG:  $F(1;15) = 5.40$ ,  $P < 0.034$ ;  $r^2 = 0.26$ ; Fig. 4A and C). Moreover, INS or TAG levels and LXR $\beta$  also showed a negative correlation (INS:  $F(1;15) = 8.02$ ,  $P = 0.013$ ;  $r^2 = 0.35$ ; TAG  $F(1;15) = 11.89$ ,  $P = 0.004$ ;  $r^2 = 0.44$ ; Fig. 4B and D).

By contrast, when AUC values were compared with LXR expressions, a significant correlation was observed only with LXR $\beta$  when the groups (CG and FG) were analyzed separately (Fig. 5D and F). Interestingly, the slopes obtained showed a negative correlation in the CG (Fig. 5D;  $F(1;5) = 10.63$ ,  $P = 0.022$ ;  $r^2 = 0.68$ ) and a positive correlation in the FG (Fig. 5F;  $F(1;8) = 5.43$ ,  $P = 0.048$ ;  $r^2 = 0.41$ ), indicating an inverse receptor behavior in the experimental condition. Further analysis evaluating the rate LXR $\beta/\alpha$  showed a significant difference (CG =  $0.96 \pm 0.07$ , FG =  $0.60 \pm 0.06$ ;  $F(1;15) = 13.95$ ,  $P = 0.002$ ) between the FG and the CG. This result indicates that even though LXR $\alpha$  expression did not show a correlation with AUC, it is





**Figure 3** Representative confocal microscopy images showing the immunoreactivity for LXR $\alpha$  and LXR $\beta$  in different hypothalamic nuclei: (A) mPOA, (B) PVN, (C) VMN, and (D) arcuate (ARC) nucleus from control animals (upper row) and fructose-treated animals (lower row). Data were obtained from four independent assays ( $n=3$  per group). LXR $\alpha$  was labeled with Alexa Fluor 546 red fluorescence and LXR $\beta$  with Alexa Fluor 488 green (2) fluorescence (first and second columns respectively). The third column shows nuclei stained with DAPI. Bar size=40  $\mu$ m. (E) Images showing Fluor 546 red (1) and Alexa Fluor 488 green (2) fluorescence when primary antibody (LXR $\alpha$  or LXR $\beta$ ) was omitted. (3, 4, and 5) Low-magnification pictures indicating the region of each hypothalamic nuclei magnified in A, B, C, and D, identified by DAPI staining. Bar size=500  $\mu$ m. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-12-0088>.

involved in the differences observed between the FG and the CG. No significant correlations among the plasma parameters determined with the hepatic LXR $\alpha$  were found.

## Discussion

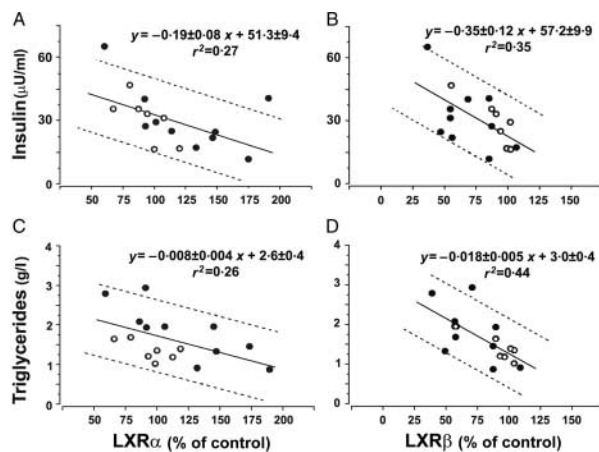
Animals with fructose in their drinking water for 6 weeks developed hypertriglyceridemia, hyperinsulinemia, and became glucose intolerant, as it was previously described in Sprague Dawley rats (Sleder *et al.* 1980, Zavaroni *et al.* 1980). These animals presented with an upregulation of LXR $\alpha$  and downregulation of LXR $\beta$  in the hypothalamus but not in the neocortex, hippocampus, or cerebellum. Moreover, we found a tight correlation between hypothalamic LXR expressions and TAG and INS levels or AUC values, indicating that these receptors are important in glucose and lipid homeostasis.

Negative correlations were found between TAG or INS and LXR $\alpha$  or  $\beta$  levels for all animals when pooled together, suggesting that a decrease in LXR expression in the hypothalamus is associated with a rise of TAG or INS levels and an increased risk of developing metabolic diseases (Alberti & Zimmet 1998, Alberti *et al.* 2005). LXR $\beta$  seems to be more sensitive to INS and TAG changes as their corresponding slopes are higher than the slopes for LXR $\alpha$  (Fig. 4).

By contrast, LXRs showed a different response regarding AUC values. LXR $\beta$  correlated negatively in control animals while a positive correlation was found in the FG. No correlation was observed between LXR $\alpha$  and AUC values. These findings would indicate that hypothalamic LXR $\beta$  may be involved in the capacity to regulate the hypothalamic vagally mediated insulin secretion. Further studies are required to define the relevance of hypothalamic LXRs on glucose homeostasis.

Overall, the upregulation of LXR $\alpha$  in FG suggests that sustained fructose consumption differently regulates LXR expressions by favoring LXR $\alpha$  signaling over LXR $\beta$  in the hypothalamus. There are probably different mechanisms underlying the regulation of LXR expressions in the hypothalamus. LXR $\alpha$  (but not LXR $\beta$ ) has been shown to be controlled by an autoregulatory mechanism. LXR activation increases LXR $\alpha$  expression in murine and human macrophages (Laffitte *et al.* 2001), myocytes (Cozzzone *et al.* 2006, Cruz-Garcia *et al.* 2011), and adipocytes (Ulven *et al.* 2004). This autoregulatory capacity may favor the induction of LXR $\alpha$  over  $\beta$ -signaling pathways (Ulven *et al.* 2004). Moreover, INS stimulates LXR $\alpha$  expression in rat hepatocytes by increasing the half-life of LXR $\alpha$  transcripts (Tobin *et al.* 2002). In accordance with this work, we found an increase in LXR $\alpha$  expression in the liver of FG compared with CG rats. However, this is unlikely the case in the hypothalamus where the INS increase correlated with a decrease in LXR $\alpha$  expression.

Endogenous receptor agonists can also contribute to modulation of LXR expression (Laffitte *et al.* 2001,



**Figure 4** Correlation between the hypothalamic levels of LXR $\alpha$  (A and C) or LXR $\beta$  (B and D) and the serum levels of insulin (A and B) or triglycerides (C and D) from male rats. For the regression plots, serum insulin levels were determined by quimioluminescence and serum triglycerides by spectrophotometry. LXR expression was determined by western blot ( $n=7-10$  animals/group). Each point represents the values corresponding to individual animals from at least three independent experiments. CG, open circles. FG, filled circles. Standard errors to the calculated slopes are shown in each panel. Dotted lines indicate the 95% confidence intervals. Significant correlation was found in the four comparisons (A:  $F(1;15)=5.4$ ,  $P=0.034$ ; B:  $F(1;15)=11.9$ ,  $P<0.004$ ; C:  $F(1;15)=5.5$ ,  $P=0.033$ ; D:  $F(1;15)=8.0$ ,  $P=0.013$ , ANOVA).

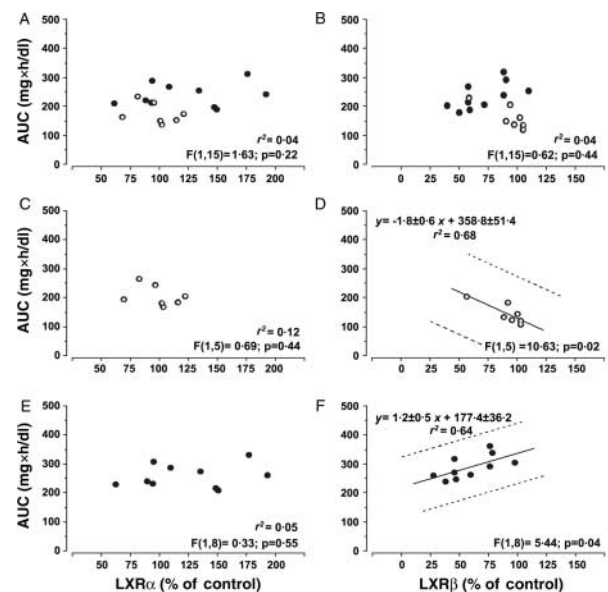
Whitney *et al.* 2001, Li *et al.* 2002, Kase *et al.* 2007). The brain produces most of the 24(S)-hydroxycholesterol present in the body, a cholesterol metabolite that acts as an efficient LXR agonist (Lutjohann *et al.* 1996, Bjorkhem *et al.* 1998). The enzyme responsible for its production is the cholesterol-24-hydroxylase and it can be induced by oxidative stress (Ohyama *et al.* 2006). On the other hand, oxidative stress was shown to be increased in high-fructose diet animals (Lin *et al.* 2011). In this way, a high-fructose diet could be affecting cholesterol-24-hydroxylase and 24(S)-hydroxycholesterol levels in the brain.

More recently, glucose has been described as another LXR agonist, inducing the expression of LXR target genes at physiological concentrations (Mitro *et al.* 2007). Both D-glucose and D-glucose-6-phosphate are more potent agonist on LXR $\beta$  than  $\alpha$  (Mitro *et al.* 2007); however, these data are controversial and could not be replicated by others (Denechaud *et al.* 2008). In this study, we found that FG animals were hyperglycemic (Table 1). Thus, the changes observed in the hypothalamic LXRs may also be related to altered levels of glucose induced by a high-fructose diet.

The hypothalamus coordinates many complex homeostatic mechanisms. LXR $\alpha$  was found to be expressed in the PVN and VMH while LXR $\beta$  was presented in the ARC nucleus. Both LXRs were found in the mPOA. Within these nuclei, there are responsive neurons to adiposity (insulin) or nutrient-related signals (glucose and fatty acids) that induce neurochemical

responses that regulate energy homeostasis (Bantubungi *et al.* 2011). Some of these signals (i.e. insulin and fatty acids) are also LXR activators/modulators (Baranowski 2008). In this context, very little is known about the function of LXRs in the brain. It was recently shown that LXR agonist stimulates genes involved in cholesterol homeostasis in the cerebellum, hippocampus, and astrocytes (Whitney *et al.* 2002). In the hypophysis, LXR agonist increases pro-opiomelanocortin expression (Matsumoto *et al.* 2009) and ACTH expression (Nilsson *et al.* 2007). In this study, we found that LXR expression is affected in glucose intolerant animals; however, these changes do not necessarily translate to changes in LXR activity. Future studies in our laboratory will focus on characterization of the LXR target genes involved and the LXR-modulated responses of hypothalamic neurons.

To our knowledge, this is the first study showing that hypothalamic LXR expression correlates with INS, TAG, and AUC levels, strongly suggesting a role of hypothalamic LXRs in lipid and glucidic homeostasis. The identification of the CNS, particularly the hypothalamus, in the control of peripheral metabolism opens a new framework to take into account when studying pathophysiologies such as obesity, metabolic syndrome, and type 2 diabetes.



**Figure 5** Correlation between the area under the curve from the glucose tolerance test (AUC) and the hypothalamic levels of LXR $\alpha$  (A, C, and E) or LXR $\beta$  (B, D, and F) in male rats. For the regression plots, the AUC was calculated using the trapezoidal method of integration (Scion Image Software, NIH) and LXR expression was determined by western blot ( $n=7-10$  animals/group). Each point represents the values corresponding to individual animals from at least three independent experiments. CG, open circles. FG, filled circles. Dotted lines indicate the 95% confidence intervals. Significant correlation was found between AUC and LXR $\beta$  in both groups when they were analyzed independently. Standard errors to the calculated slopes are shown in D and F. Correlation coefficient and ANOVA data are shown at the lower right side of each panel.

## 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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