Sex differences in LXR expression in normal offspring and in rats born to diabetic dams

María Sol Kruse[1], María Cristina Vega[1], Mariana Rey[1] and Héctor Coirini[1,2,3]

[1]Laboratorio de Neurobiología, Instituto de Biología y Medicina Experimental (IByME-CONICET), Vuelta de Obligado 2490, C1428ADN Ciudad Autónoma de Buenos Aires, Argentina
[2]Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 5to Piso, C1121ABG Ciudad Autónoma de Buenos Aires, Argentina
[3]Facultad de Ciencias Medicas, Universidad Católica de Cuyo, Rivadavia, Provincia de San Juan, Argentina

Correspondence should be addressed to H Coirini
Email
hcoirini@ibyme.conicet.gov.ar

Abstract

Gestational diabetes (GD) alters normal fetal development and is related to a diabetogenic effect in the progeny. Liver X receptors (LXRs) are considered to be potential drug targets for the regulation, treatment, or prevention of diabetes. The aim of this study was to evaluate early and late changes of LXR in the hippocampus and hypothalamus of the male and female offspring of control (CO) and diabetic (DO) mothers. We used an experimental model of streptozotocin-induced GD to assess the protein expression of LXRα (NR1H3) and LXRβ (NR1H2) by western blotting. The tissues were obtained from CO and DO animals at postnatal day 1 (1D), day 10 (10D), and day 35 (35D) and 9 months (9M). In CO, the LXR expression showed significant differences among the groups, which were tissue- and receptor-specific ($P<0.05$). Sex differences in CO were found only in the hypothalamus for LXRβ expression at 35D and 9M ($P<0.05$). When CO and DO were compared, differences between them were observed in the majority of the studied groups at 1D (male hippocampus, LXRα 31% and LXRβ 161%; female hippocampus, LXRβ 165%; male hypothalamus, LXRβ 182%; and female hypothalamus, LXRα 85%; $P<0.05$). However, these differences disappeared later with the exception of LXRβ expression in the male hypothalamus ($P<0.05$). The area under the curve during the glucose tolerance test correlated negatively with LXRβ in CO but not in DO animals. Moreover, in a male DO subpopulation this correlation was positive as it occurs in intolerant animals. These results indicate that GD affects hypothalamic LXR expression differently in male and female offspring.

Key Words
- glucose tolerance test
- AUC
- gestational diabetes
- insulin resistance

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Introduction

Liver X receptor α (LXRα, NR1H3) and LXRβ (NR1H2) are nuclear receptors that trigger various responses 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. The expression of these receptors is increased in pancreatic β-cells in type 2 diabetes (Choe *et al.* 2007), and LXR stimulation normalizes glycemia, thus improving insulin sensitivity in rodent models of type 2 diabetes (Cao *et al.* 2003, Laffitte *et al.* 2003, Commerford *et al.* 2007) without affecting glycemia in nondiabetic animals (Cao *et al.* 2003, Laffitte *et al.* 2003).

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Both LXR subtypes are present in the CNS, although the expression of the β-subtype is greater than that of the α-subtype (Schmidt *et al.* 1999, Whitney *et al.* 2002). Nevertheless, the distribution of LXR expression in the brain and their physiological function, in particular with respect to brain control of energy homeostasis, remains to be clarified.

Recently we have demonstrated that LXR expression is altered in the hypothalamus of glucose-intolerant rats. Rats fed with a fructose-rich diet for 6 weeks develop glucose intolerance, decreased LXRβ levels, and increased LXRα expression in the hypothalamus whilst no effect is observed on the LXR expression in the hippocampus, cerebellum, or neocortex (Kruse *et al.* 2012*a*). Moreover, both LXRα and LXRβ expression correlate negatively with serum levels of insulin and triglyceride. The area under the curve (AUC) during the glucose tolerance test also correlated negatively with the levels of hypothalamic LXRβ. Interestingly, the AUC–LXRβ correlation is altered in intolerant rats, indicating that the hypothalamus, through this subtype, is especially sensitive to glucose.

Gestational diabetes (GD) is considered a risk factor for development of type 2 diabetes and other metabolic diseases in the offspring (Silverman *et al.* 1995, Hillier *et al.* 2007). It is known that GD alters normal fetal development and has a diabetogenic effect on the progeny. We have shown that GD affects both the apoptotic and proliferation pathways in the brains of the developing offspring of diabetic rats (Kruse *et al.* 2012*a*).

In this study, we studied the expression of LXRα and LXRβ in two brain regions of control rats and rats exposed to hyperglycemia during gestation. The expression of these receptors evaluated at different developmental stages and were compared between sexes. The results of this study indicate that hypothalamic LXRβ expression, but not that of LXRα, matures differently in the two sexes. Moreover, GD induced long-term alterations in LXRβ expression in the hypothalamus in males, but not in females. In these animals, the hypothalamic LXRβ/AUC correlation was also altered compared with controls. Altogether the data indicate that male rats exposed to GD may be more susceptible to developing metabolic diseases related to LXR alterations.

Materials and methods

Experimental animals

Animal procedures were approved by the Animal Care and Use Ethical Committee of the School of Medicine, University of Buenos Aires, Argentina, 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 were freely available. Sixty-day-old female Sprague–Dawley rats weighting 210–260 g ($n=8$) were placed overnight in cages with males of the same strain. Vaginal smears were examined the next morning and the presence of spermatozoa was considered to identify day 1 of gestation. Diabetes was induced on gestational day 3 by a single femoral i.v. injection of 35 mg/kg streptozotocin (STZ, Sigma–Aldrich) dissolved in 0.9% saline acidified to pH 4.5 using citric acid ($n=4$) (Coirini *et al.* 1980). Vehicle-injected rats served as controls ($n=4$). At 48 hours after STZ administration, a pronounced glucosuria (>2 g/100 ml, Diastix; Bayer) and elevation of blood sugar levels of >180 mg/dl were detected in all rats. After delivery, pups were placed with foster mothers. Animals were then killed at different ages by decapitation. The hypothalamus and hippocampus were rapidly dissected, frozen on dry ice, and stored at −80 °C.

Glucose tolerance test

After animals had been fasted for 10 h, blood samples were collected from the tail vein and glucose levels were determined by using a commercial strip and a glucometer (OneTouch Ultra, Johnson & Johnson, Buenos Aires, 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 postinjection. The AUC during the glucose tolerance test was calculated using the trapezoidal method of integration.

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 (Kruse *et al.* 2009*a*,*b*). A total of 20 μg 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

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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), and F-actin (1:1000, Santa Cruz Biotechnology) (Kruse *et al*. 2012*b*). 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 Instiutes 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.

Statistical analysis

Values are expressed as mean±S.D. At least three 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. 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

The expression of LXRα and LXRβ in the hippocampus and hypothalamus was studied in neonatal (1D), infant (10D), juvenile (35D), and adult (9M) rats by western blot. The results were then compared with the expression of LXR in the offspring of diabetic dams (DO). GD was induced by a single dose of STZ on gestational day 3 (Kruse *et al*. 2012*a*). ANOVA analysis showed that the LXR changes during ontogeny are more drastic for LXRβ (four- to eightfold) than for LXRα (until twofold) in all groups studied (female hippocampus: $F(1,54)=47.70$, $P<0.0001$; male hippocampus: $F(1,79)=16.38$, $P<0.0001$; female hypothalamus: $F(1,54)=9.17$, $P<0.005$; and male hypothalamus: $F(1,83)=52.34$, $P<0.0001$) (Figs 1, 2, 3 and 4).

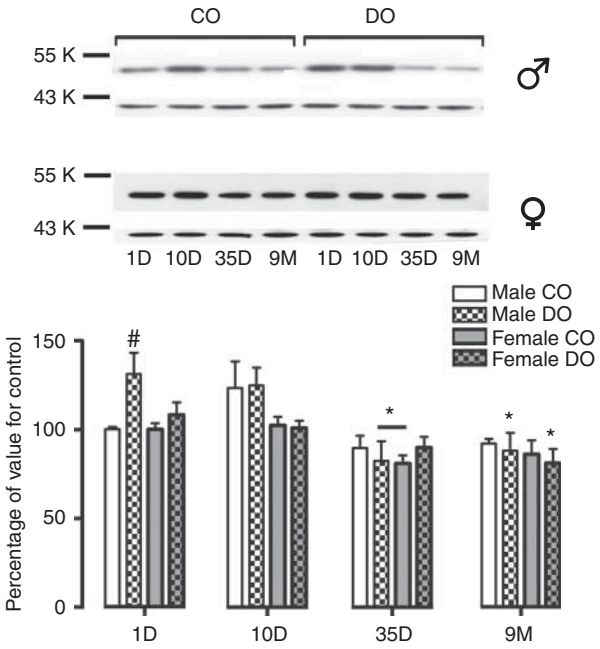

Figure 1
Western blot of LXRα in the hippocampus of male (white bars) and female (gray bars) CO (plain bars) and DO (patterned bars). Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of LXR expression and the F-actin loading control are shown in the upper panel. Data are presented as mean±S.D. from at least three independent experiments, $n=7$–13 animals/group. Significant differences between ages (*) or between CO and DO (#) were identified by one-way ANOVA followed by Fisher's *post-hoc* test. *$P<0.05$ (male 35D DO and 9M DO vs 1D DO, female 35D CO vs 1D CO, and female 9M DO vs 1D DO) and $^{\#}P<0.05$ (male 1D DO vs 1D CO).

LXR expression in the hippocampus

In the hippocampus of control offspring, LXRα expression decreased at 35D of age in females (19% ANOVA, Fisher $P<0.05$), whereas no significant differences were found in males at any age (Fig. 1). Regarding LXRβ signal, we observed two peaks for males at 10D and 9M in the hippocampus (209 and 178% respectively; $P<0.05$) and a significant increase in females at 9M in the hippocampus (193%; $P<0.05$) (Fig. 2). Statistical analysis showed no differences between sexes (LXRα: $F(1,35)=2.65$, $P=0.11$ and LXRβ: $F(1,42)=0.025$, $P=0.87$).

The LXR expression levels in offspring of control rats (CO) were then compared with those of rats born to diabetic mothers (DO). We found a significant increase of both LXRα/β expression at 1D (LXRα male hippocampus: 31%, $P<0.05$; LXRβ female hippocampus: 165%, $P<0.05$; and LXRβ male hippocampus: 161%, $P<0.005$), indicating that DO at 1D is still affected by the exposure to hyperglycemia during gestation (Figs 1 and 2). No further LXR differences between CO and DO were detected at other ages.

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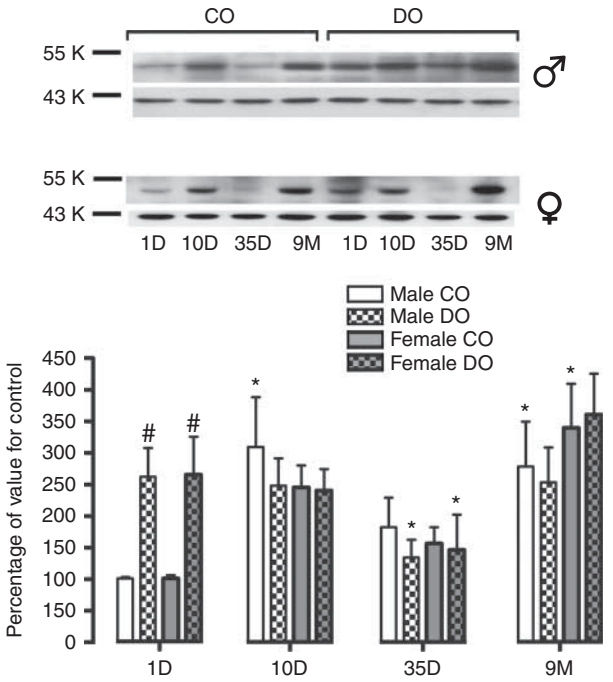

Figure 2
Western blot of LXRβ in the hippocampus of male (white bars) and female (gray bars) CO (plain bars) and DO (patterned bars). Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of LXR expression and the F-actin loading control are shown in the upper panel. Data are presented as mean±S.D. from at least three independent experiments, $n=7$–13 animals/group. Significant differences between ages (*) or between CO and DO (#) were determined by one-way ANOVA followed by Fisher's *post-hoc* test. *$P<0.05$ (male 10D CO and 9M CO vs 1D CO, male 35D DO vs 1D DO, and female 9M CO vs 1D CO and 35D DO vs 1D DO) and #$P<0.05$ (male and female, 1D DO vs 1D CO).

LXR expression in the hypothalamus

In the hypothalamus, there was a 63% increase in LXRα expression at 9M of age in males ($P<0.05$) and a 65% increase at 35D in females ($P<0.05$) (Fig. 3). LXRβ expression showed a peak at 9M of age in males (796%, $P<0.0001$) and two peaks at 10D and 9M in females (298%, $P<0.01$ and 342%, $P<0.005$ respectively). Sex differences were only found for LXRβ expression in adults (LXRβ 35D and 9M, Student's *t*-test $P<0.05$; LXRα: $F(1,39)=0.002$, $P=0.97$) (Fig. 4).

When CO was compared with DO, we found a significant increase at 1D in female LXRα (85%, $P<0.05$) and in male LXRβ (182%, $P<0.005$) levels (Figs 3 and 4). These differences disappeared later in life except for the hypothalamus in males where LXRβ expression dropped (9M, CO 896% vs DO 573%, Student's *t*-test $P<0.05$) (Fig. 4). Sex differences were found for LXRβ expression at 35D (Student's *t*-test $P<0.05$). At 9M the LXRβ difference observed between males and females in control hypothalamus disappeared in DO (Fig. 4).

All these results indicate that GD affects males and females differently, having long-term consequences only in the hypothalamus of adult DO males.

Glucose tolerance test in adult CO and DO

The ability to regulate a glucose load was tested in 5-month-old (5M) adult rats as DO animals over that age start to develop glucose intolerance (Boloker *et al.* 2002). As with 9M animals, 5M rats showed decreased LXRβ expression in DO males (26%, $P<0.05$) but not in females. After i.p. injection of glucose solution (2 g/kg), two subpopulations were distinguished in the DO group. A total of 38% of male and 36% of female DO animals displayed glucose intolerance, showing significant changes at 30, 60, and 120 min (Fig. 5). The AUC during the glucose tolerance test was then calculated using the

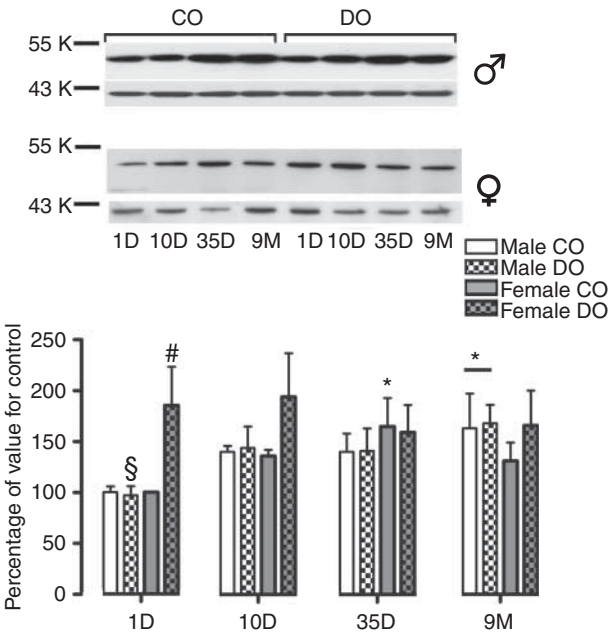

Figure 3
Western blot of LXRα in the hypothalamus of male (white bars) and female (gray bars) CO (plain bars) and DO (patterned bars). Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of LXR expression and the F-actin loading control are shown in the upper panel. Data are presented as mean±S.D. from at least three independent experiments, $n=7$–13 animals/group. Significant sex differences (§) and differences between ages (*) or between CO and DO (#) were determined by one-way ANOVA followed by Fisher's *post-hoc* test. *$P<0.05$ (male 9M CO vs 1D CO, male 9M DO vs 1D DO, and female 35D CO vs 1D CO), #$P<0.05$ (female 1D DO vs 1D CO), and §$P<0.05$ (male 1D DO vs female 1D DO).

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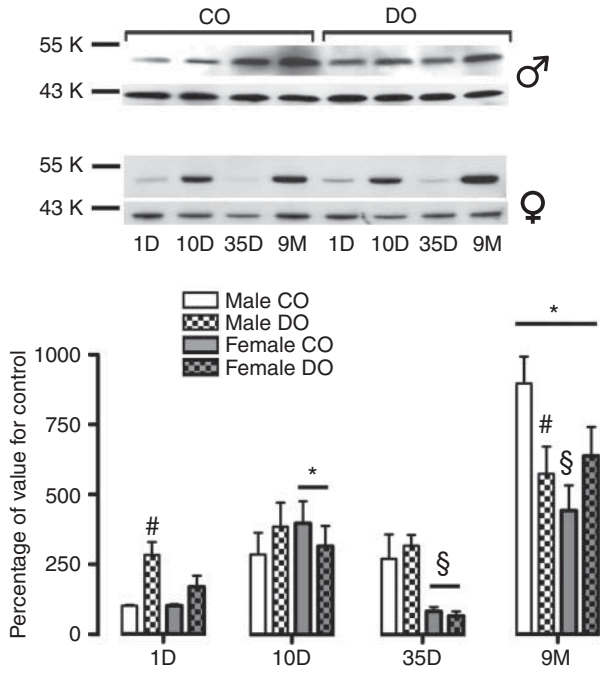

Figure 4
Western blot of LXRβ in the hypothalamus of male (white bars) and female (gray bars) CO (plain bars) and DO (patterned bars). Data were quantified by densitometric analysis and corrected with reference to the F-actin loading control. Representative pictures of LXR expression and the F-actin loading control are shown in the upper panel. Data are presented as mean±S.D from at least three independent experiments, $n=7$–13 animals/group. *Significant sex differences (§) and differences between ages (*) or between CO and DO (#) were determined by one-way ANOVA followed by Fisher's *post-hoc* test. $^{\#}P<0.01$ (male 1D DO vs 1D CO and 9M DO vs 9M CO), $^{*}P<0.01$ (male 9M CO vs 1D CO, 9M DO vs 1D DO, female 10D and 9M CO vs 1D CO, and 10D and 9M DO vs 1D DO), and $^{\S}P<0.05$ (male 35D CO vs female 35D CO, male 35D DO vs female 35D DO, and male 9M CO vs female 9M CO).

trapezoidal method of integration (Kruse *et al*. 2012*b*). The glucose-intolerant animals presented an AUC significantly higher than CO animals and DO animals that did not develop glucose intolerance (animals with AUC >300 vs animals with AUC <300 respectively; Fig. 5, inset).

Correlation between LXRβ expression and AUC

In a previous work, we have shown that the AUC correlated negatively with the hypothalamic LXRβ levels but not with LXRα levels. Moreover, in an animal model of glucose intolerance, LXRβ showed a positive correlation with AUC, indicating an inverse receptor behavior under these experimental conditions (Kruse *et al*. 2012*b*).

In this study, we compared the correlation curves between AUC and the hypothalamic LXRβ levels in 5M CO and DO animals. In accordance with our previous study, we observed a negative correlation between the AUC and male hypothalamic LXRβ levels in CO (Fig. 6A). The slope of the curve obtained was similar to the one we had previously observed in CO animals at three months of age (Kruse *et al*. 2012*b*). In female CO, the same correlation was found (Fig. 6B). The situation was different in DO animals. When we combined all the animals together, no significant correlation was found (Fig. 6C and D). However, when we separated the animals into two different populations on the basis of their AUC values (glucose-tolerant animals AUC <300 or glucose-intolerant animals AUC >300), two kinds of regressions were obtained (Fig. 7). Male animals that had AUC value below 300 showed a negative AUC–LXRβ correlation (Fig. 7A), while animals with AUC over 300 presented a positive AUC–LXRβ correlation (Fig. 7C). In contrast, females with AUC below 300 showed, like controls, a negative AUC–LXRβ correlation (Fig. 7B), while animals with AUC over 300 did not display any correlation (Fig. 7D).

Discussion

In this study, we found that LXRβ, but not LXRα, is altered in the hypothalamus of adult male offspring born to diabetic dams. In contrast, female offspring did not show long-term LXR changes when compared with controls. No changes were observed between CO and DO in the hippocampus in both males and females. Moreover, the correlation between AUC and hypothalamic LXRβ levels is positive in a subpopulation of adult male DO (Fig. 7C), indicating that there is a population in this group capable of developing glucose intolerance associated with altered hypothalamic LXRβ expression. In contrast, female DO did not show any positive LXRβ–AUC correlation (Fig. 7D).

It is now widely accepted that intrauterine exposure to maternal diabetes alters metabolism and increases the risk of obesity and type 2 diabetes in the offspring, in addition to genetic predisposition, regardless of maternal diabetes type (Dabelea 2007). However, the underlying mechanisms by which exposure to diabetes in the uterus increases the risk of offspring obesity are not fully understood. It has been proposed that untreated diabetes in pregnant rats leads to 'malprogramming' of the hypothalamic neuropeptidergic neurons in offspring, leading to increased orexigenic neuropeptide Y and agouti-related peptide, which could contribute to hyperphagia and later development of overweight (Franke *et al*. 2005).

In this context, we speculate that the LXRβ alterations observed principally in male DO would probably affect responses of hypothalamic neurons related to energy balance and glucose homeostasis. Little is known about

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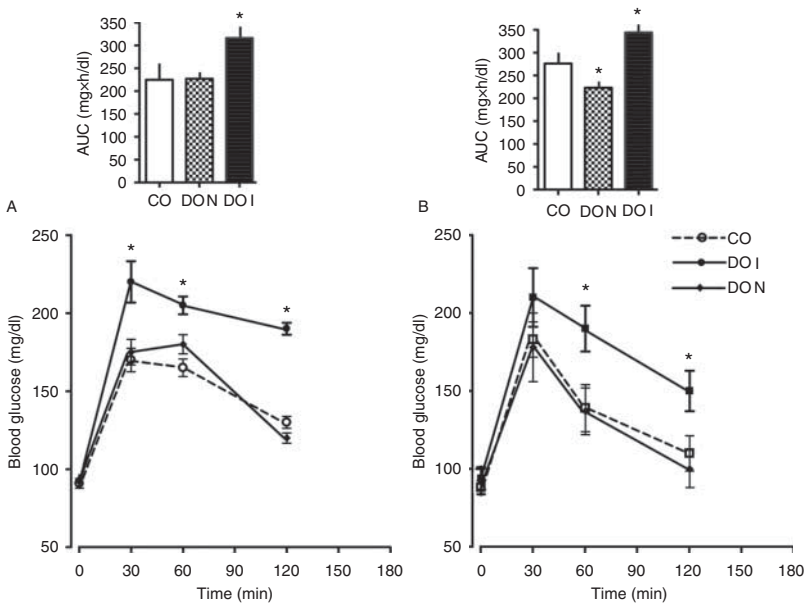

Figure 5
Curves of glucose tolerance in CO and DO. The animals were fasted for 10 h and after the first sampling $t=0$ they received i.p. injections of a glucose solution (2 g/kg body weight). Blood samples were drawn from the tail vein at 30, 60, and 120 min after the glucose load. (A) Males. (B) Females. Inset: Numerical integration of the glucose tolerance curve (AUC). DO N, DO animals with AUC <300 and DO I, DO animals with AUC >300. Males: $F(2,17)=4.21$, $P=0.033$ and females: $F(2,13)=9.37$, $P=0.030$ ($n=4$–7 animals/group), $^*P<0.05$.

the function of LXR in the hypothalamus. It has been shown that LXRβ$^{-/-}$ but not LXRα$^{-/-}$ mice lose arginine vasopressin production, in magnocellular neurons of the paraventricular nucleus of the hypothalamus. These animals exhibit polyuria and polydipsia, both features of diabetes insipidus (Gabbi *et al.* 2012). In a previous work we examined LXR expression in different nuclei of the hypothalamus. The paraventricular and ventromedial nuclei express mainly LXRα, whereas the arcuate nucleus expresses LXRβ. Both LXR are present in the median preoptic area (Kruse *et al.* 2012*b*). Future studies at our laboratory will focus on elucidating whether LXR is capable of affecting hypothalamic responses.

In this paper, we found that at one day of age most of the DO groups presented an increase in LXR expression, indicating that LXR may still be affected by hyperglycemia at that age. During development, LXR plays a pivotal role in the migration of cortical neurons (Fan *et al.* 2008). If LXR exerts the same effect in other brain areas (hippocampus and hypothalamus), the alterations

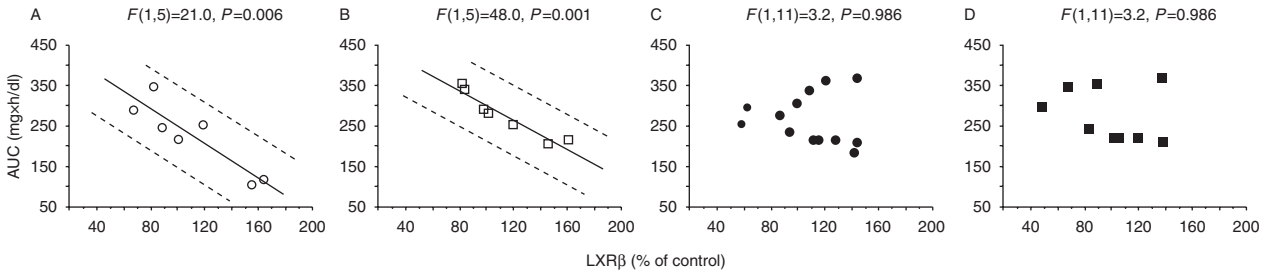

Figure 6
Correlation between the area under the curve (AUC) from the glucose tolerance test and the hypothalamic levels of LXRβ in males (A and C, circles) and females (B and D, squares). For the regression plots, the AUC was calculated using the trapezoidal method of integration (Scion Image Software, NIH) and LXR expression was determined by western blot ($n=7$–13 animals/group). Each point represents the values corresponding to individual animals from at least three independent experiments (CO, open points and DO, filled points). Significant correlation was found between AUC and LXRβ in control groups but not in diabetic offspring. One-way ANOVA data are shown for each panel. Unbroken lines show the correlations and dotted lines indicate the 95% confidence intervals.

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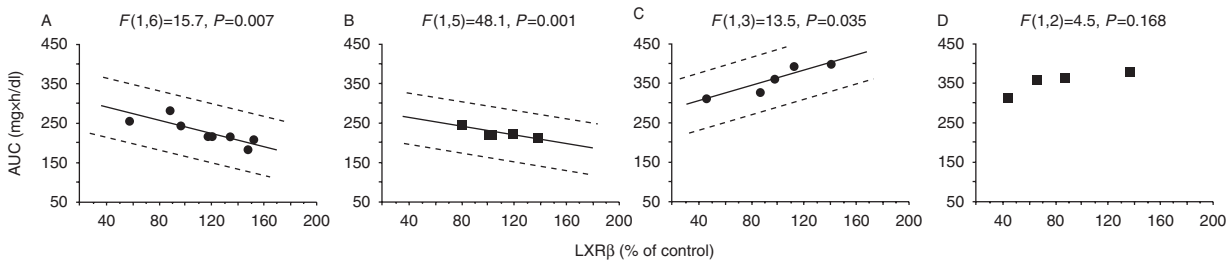

Figure 7
Correlation between the area under the curve (AUC) from the glucose tolerance test and the hypothalamic levels of LXRβ in male DO (A and C, circles) and female DO (B and D, squares). The AUC was calculated using the trapezoidal method of integration (Scion Image Software, NIH) and LXR expression was determined by western blot. Each point represents the values corresponding to individual animals from at least three independent experiments ((A) and (B), animals with AUC <300 and (C) and (D), animals with AUC >300). One-way ANOVA data are shown for each panel. Unbroken lines show the correlations and dotted lines indicate the 95% confidence intervals.

observed in DO may influence their brain cytoarchitecture. Indeed, the migration of the neurons from the neuroepithelium in the hypothalamus is controlled by Notch effector Hes1 (Aujla *et al.* 2011), among other factors, and this pathway appears to be regulated by LXR (Kim *et al.* 2010).

In a recent study, we showed that uncontrolled GD disrupts both neuronal proliferation and neuronal survival in nonmalformed rat embryos at GD19. This is not associated with changes in GFAP levels and heavy neurofilament expression (e.g. NF-200) in the brain from offspring of diabetic rats, indicating that the total number of neurons or glia is not affected by GD at this age (Kruse *et al.* 2012*a*). However, since cell proliferation combined with apoptosis sculpts the developing CNS (i.e. pruning), it is expected that there would be enduring neurobiological consequences in the adult brain of DO. In this study, we found at least one long-term effect triggered by GD. Adult male DO presented lower expression of LXRβ in the hypothalamus compared with CO at the same age. Moreover, GD increases the frequency of glucose-intolerant animals in both sexes, which in our assay was 38% for males and 36% for females in 5M old animals (Fig. 5). Those animals presented increased AUC and an altered AUC–LXRβ correlation. Even though we found the same proportion of intolerant animals, male DO seem to be more affected by the hyperglycemic state during development. Adult male DO was the only group showing a significant decrease in LXRβ receptor expression and a subpopulation of this group showed a shift of the AUC–LXRβ correlation curve from negative to positive, as observed previously in a different model of glucose intolerance. In this model, rats subjected to a fructose-rich diet for 6 weeks developed hypertriglyceridemia and hyperinsulinemia and became glucose intolerant, indicating a progression toward type 2 diabetes. These animals present a decreased hypothalamic LXRβ expression while showing no LXR changes in other brain areas (hippocampus, cerebellum, and neocortex). The situation is different in female DO animals. No long-term LXR changes were found, and even though the AUC–LXRβ correlation was altered in DO compared with CO, no positive correlation was found in this group.

It seems possible that significant sex difference in glucose tolerance rates appears as the animals become older. Male rats gain body weight more rapidly than females, and adipose tissue is preferentially distributed in the abdominal or visceral region (male pattern of body fat distribution). This distribution carries a much greater risk for metabolic disorders than does adipose tissue distributed subcutaneously (female pattern; Wajchenberg 2000). Ovariectomized rats gain visceral fat with no change in subcutaneous fat (Clegg *et al.* 2006). Peripheral or central administration of estradiol to these rats restores central leptin sensitivity and changes their body fat distribution to mirror that of intact females. These findings indicate that estrogen regulates body fat distribution. The relative visceral fat volume increases with age more in males than in females (Kotani *et al.* 1994), indicating that there is a sex difference in the age-related changes in whole-body fat distribution, especially in the abdominal fat tissues. Moreover, male sex is a risk factor for unfavorable perinatal outcome (Grill *et al.* 1991), and those hyperglycemic levels of the mother could result in different effects on the offspring (Regnault *et al.* 2013). Altogether these results indicate that GD induces different changes depending on the sex, rendering the male progeny more susceptible to developing glucose intolerance and metabolic disturbances related to LXR alterations.

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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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