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Perinatal programming of the orexinergic (hypocretinergic) system in hypothalamus and anterior pituitary by testosterone

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ABSTRACT

Orexins A/B derived from hypothalamic prepro-orexin (PPO) are agonists for orexin receptors 1 (OX1) and 2 (OX2). Previously, we showed clear sex differences in the hypothalamic-pituitary-gonadal orexinergic system in adult rodents. Here, we studied the effect of sexual brain differentiation on the orexinergic system in neuroendocrine structures regulating reproduction. We evaluated: a: proestrous and neonatally androgenized female rats; b: adult males, untreated or gonadectomized in adulthood and injected with oil or estradiol and progesterone (E_2/P_4); c: control and demasculinized males (perinatally treated with flutamide and later castration) injected either with oil or E_2/P_4 in adulthood. Rats were sacrificed at 12:00 and 18:00 h; blood samples and brains were collected. Hormones were measured using radioimmunoassay. PPO, OX1 and OX2 mRNAs were quantified by qPCR in medial basal hypothalamus, anterior hypothalamus, adenohypophysis, and cortex. Western blots for OX1 were done in the same structures. In normal females, gonadotropins surged at 18:00 h coinciding with significant elevations of PPO, OX1 and OX2 mRNAs and OX1 protein in hypothalamus and pituitary; no increases were observed at noon. Afternoon changes were absent in masculinized females. Demasculinized males when treated with E_2/P_4 showed high PPO, OX1 and OX2 mRNAs and OX1 protein expression in hypothalamus and pituitary at 12:00 and 18:00 h compared vehicle-treated controls. The same steroid treatment was ineffective in males with normal brain masculinization. Here we show that neonatal testosterone shapes the sexual differences in the hypothalamic-pituitary orexinergic system in synchronicity to establishing the brain sex differences of the reproductive axis. The female brain controls gonadotropin surges and concurrent elevations of all studied components of the orexinergic system, suggesting its participation as a possible link between food intake, behavior and hormonal control of reproduction.

1. Introduction

The neuroendocrine systems play critical roles in the regulation of vital functions, such as food intake and reproduction. Mechanisms controlling reproduction depend decisively on food availability. Many examples from physiology and pathology indicate that reproduction, food intake and energy balance are tightly integrated with each other and with the inner clock and alertness. Physiological examples of reproductive changes correlating with food intake are: puberty, with the inherent increase of food intake and body mass in both sexes; estrous/menstrual cycles, presenting peri/postovulatory food intake decrease; pregnancy and lactation, with the characteristic positive energy balance in the female. Clinical examples of reproductive system dysfunctions are conditions such as obesity, associated to menstrual, ovulatory and fertility alterations; loss of body mass from several etiologies correlated with menstrual and ovulatory failure; anorexia, associated to anovulation [1].

A question that arises from those observations is how reproduction and energy homeostasis are integrated, and which regulatory molecules participate in integrating these functions [2,3]. Among other molecules involved in this regulation, orexin A and orexin B (hypocretins A and B) are neuropeptides derived by proteolytic cleavage from a 130 amino acid precursor, prepro-orexin (PPO), which was originally isolated from the rat hypothalamus [4,5]. They are synthesized mainly by neurons with their soma located in the lateral hypothalamus and projections throughout the brain. Both peptides are agonists for the G protein-coupled orexins receptors 1 (OX1) and 2 (OX2). The structure of orexins and their receptors is highly conserved in mammals including rodents and humans. Both receptor genes are widely expressed within the rat brain, but with some differences in the OX1 and OX2 distribution; furthermore, differential roles for OX1 and OX2 receptors have been suggested [6–10].

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Functionally, orexins have been related to regulation to appetite, food intake and feeding behavior, sleep, arousal and alertness, and to some neuroendocrine functions, such as reproduction, including modulation of sexual behavior [11–13]. Their participation in the brain control of the pituitary secretion, including gonadotropins, was postulated in rodents and humans [14–23]. Orexins have also been shown to modulate GnRH secretion directly [16,19,23]. In addition, sex differences in PPO and OX1 expression levels were reported in hypothalamus (PPO: females > males) [22] and pituitary (OX1: males > females) [21].

Previously, we demonstrated clear differences between the female and male adult rat orexinergic systems in the hypothalamic-pituitary-gonadal unit. Furthermore, we showed the participation of the orexinergic system in the neuroendocrine events leading to ovulation. In females PPO, OX1 and OX2 mRNA expression increases in late proestrus in hypothalamus and anterior pituitary but not in cortex. This cyclic and circadian variation is absent in males. In addition, orexin receptor antagonists administered during proestrus were able to decrease the preovulatory gonadotropin surges and reduced ovulation. Moreover, we have investigated the impact of orexins on the regulation of pituitary secretion, and conversely, the impact of the sexual hormonal milieu on the hypothalamic-pituitary orexinergic system in adulthood [24–27].

It is known that brain-controlled reproductive functions differ between males and females and that the perinatal environment is critical for programming the mechanisms responsible for the metabolic control of reproduction. Food deficits inhibit the reproductive axis, and this is particularly important for females since they need extra energy during gestation and lactation [28]. Sexually dimorphic differences in neuronal connections and brain structures are the result of the irreversible action of gonadal hormones during a sensitive perinatal period. In rats, androgens secreted both prenatally by fetal testis and early postnatally permanently masculinize and defeminize neural substrates, controlling sexually dimorphic brain functions, behavior and genital morphology. Conversely, steroid effects on female gonadotropin patterns and sexual behavior are primarily postnatal. These sexually divergent anatomical and behavioral characteristics are the consequence of changes arising from turning on or shutting off gene expression, e.g. reflected by differences in receptor or enzyme levels [29–35].

Due to the clear sexually dimorphic expression of the orexinergic system in central neuroendocrine structures regulating reproduction such as the hypothalamus and pituitary, the aim of the present work was to discriminate between the hormonal milieu of proestrus and the sexual differentiation of the brain as the origin of this particular expression pattern.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room (21 °C), with lights on from 07:00 h to 19:00 h. They were given free access to laboratory chow and tap water. Experimental procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IBYME-CONICET), in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health, Animal Welfare Assurance for the Institute of Biology and Experimental Medicine A#5072-01.

Adult female rats were mated on proestrus and day 1 of gestation was considered when a vaginal sperm plug was noted the next day. On the day of birth (designated day 1) the sex of the pups was determined by anogenital distance, as described [33,36].

We studied normal and masculinized adult females, normal and demasculinized adult males and also males orchidectomized at adulthood.

2.1.1. Control and masculinized females

Newborn females were injected subcutaneously (s.c.) on the day of birth either with 0.05 ml castor-oil as control or with a single injection of 100 μg of testosterone propionate in 0.05 ml castor oil (neonatally androgenized females: TP) [33]. Females were studied at adulthood [body weight (BW): 200–250 g]. Control females were cycled and sacrificed in proestrus after two regular cycles, at two different times of day: 12:00 and 18:00 h. Androgenized females did not cycle and were sacrificed at the same times as cycling controls.

The goal was to determine whether androgenization had an impact on the gonadotropic surges and their correlation with the expression of prepro-orexin (PPO), orexins receptors 1 (OX1) and 2 (OX2), in hypothalamus, cortex (as control tissue) and anterior pituitary.

2.1.2. Control and orchidectomized adult males with or without E_2-P_4 treatment

A group of males was left untreated until adulthood. Two weeks before sacrifice they were either sham operated (Cont) or orchidectomized (Gx). Two days before sacrifice both groups were divided in two subgroups. One subgroup of each group was injected with castor oil (Cont; Gx), and the second subgroup was injected with estradiol (E_2) and progesterone (P_4) (Cont-E_2P_4; Gx-E_2P_4), see below.

For steroid treatment, 2 days before sacrifice male rats were injected at 09:00 h with 10 μg/kg BW of 17β Estradiol (Sigma-Aldrich, St. Louis, MO, USA) and 48 h later they received, at 09:00 h, a dose of 1.5 mg/kg BW of progesterone (Sigma–Aldrich).

2.1.3. Control and demasculinized males with or without E_2-P_4 treatment

Timed pregnant rats were s.c. daily-injected with either 10% ethanol in castor oil for controls or flutamide. Flutamide (2-methyl-*N*-[4-nitro-3-(trifluoromethyl)-phenyl] propanamide; Sigma–Aldrich St. Louis, MO, USA), an androgen receptor antagonist that blocks testosterone (T) and dihydrotestosterone action [36–40], was administered at 25 mg/kg BW, and adjusted daily based on the BW of the dams; it was prepared by dissolving the drug completely, first in absolute ethanol and then diluting it in castor oil. Flutamide was injected from gestational day 17 until the day before delivery to abolish the effects of the prenatal T peak.

Newborn male pups from oil-treated mothers were treated with oil (Cont). Newborn male pups from flutamide-treated mothers were s.c injected with the same dose of flutamide (25 mg/kg, 10 μl/10 g BW) on postnatal days 1, 3, 5, 7 and 9. In addition, on postnatal day 11, they were orchidectomized under cold anesthesia (Flut-Gx).

The goal of this group was to prevent the masculinization of the brain due to the pre and postnatal T peaks, through the treatment with the antiandrogen flutamide and later castration.

At adulthood, 2 days before sacrifice control males were injected with castor oil (Cont) while demasculinized males were injected with either castor oil (Flut-Gx) or E_2 and P_4 (Flut-Gx-E_2P_4), as above.

The aim of the sequential treatment with E_2 and P_4 was used as a model to trigger gonadotropic surges in demasculinized males, which resembled the FSH and LH surges observed in normal females during proestrous afternoon [33].

In all cases, animals were sacrificed by decapitation (control females in proestrus) at 1200 or 1800 h and blood and tissue samples were collected.

2.2. Hormone determinations

Serum FSH and LH were determined by RIA using kits obtained through NHPP, NIDDK and Dr. A. Parlow, as previously described [27]. Results were expressed in terms of RP3 rat FSH and LH standards. Assay sensitivities were 0.1175 ng/ml for FSH and 0.015 ng/ml for LH. Intra- and inter-assay coefficients of variation were for FSH 8.0% and 13.2%,

respectively, and for LH 7.2% and 11.4%, respectively.

Serum E_2, P_4 and T were determined by RIA using specific antisera kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO, USA) after ethyl ether extraction, as previously described [25,26]. Labeled E_2, T and P_4 were purchased from PerkinElmer (Wellesley, MA, USA). Assay sensitivities were 11.3 pg for estradiol, 500 pg for progesterone and 12.5 pg for testosterone. Intra-and inter-assay coefficients of variation were 6.8 and 11.7% respectively for estradiol, 7.1 and 12.15% respectively for progesterone and 7.8% and 12.3% respectively for testosterone.

2.3. Tissue samples

Brains were rapidly removed and placed on ice for dissection. An area limited anteriorly by the cephalic fissure of the optic chiasm, laterally by the hypothalamic fissures, posteriorly by the fissure caudal to the mammillary bodies and in depth by the subthalamic sulcus was excised. A transversal section through the insertion of the optic chiasm divided the hypothalamus in two: the medial basal-mammilary region (MBH) and the anterior-preoptic suprachisamatic area (AH). Anterior pituitary (Pit) and frontoparietal cortex (Ctx), as a control brain area, were also removed; all tissue samples were immediately homogenized in TRIzol reagent (Invitrogen, CA, USA) and kept at −70 °C until used [24,25].

2.4. Total RNA preparation and cDNA synthesis

Total RNA was isolated from tissue homogenates by use of the TRIzol reagent method. The RNA concentration was determined on the basis of absorbance at 260 nm, and its purity was evaluated by the ratio of absorbance at 260/280 nm (> 1.8). RNAs were kept frozen at −70 °C until analyzed.

After digestion of genomic DNA by treatment with deoxyribonuclease I (Ambion, Austin, TX), first-strand cDNA was synthesized from 1 µg of total RNA in the presence of 10 mM MgCl2, 50 mM Tris-HCl (pH 8.6), 75 mMKCl, 0.5 mM deoxy-NTPs, 1 mM DTT, 1 U/µl Rnase OUT (Invitrogen, Argentina), 0.5 µg oligo(dT)15 primer (Biodynamics, Buenos Aires, Argentina), and 20 U of MMLV reverse transcriptase (Epicentre, Madison, WI). To validate successful deoxyribonuclease I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

2.5. Quantitative real-time PCR

Sense and antisense oligonucleotide primers were designed based on the published cDNA sequences for PPO, OX1-R, OX2-R, and cyclophilin using the Primer Express software (Applied Biosystems, Foster City, CA), as published [27]. Oligonucleotides were obtained from Invitrogen, Argentina. The sequences of the primers are as follows:

PPO sense GCCTCAGACTCCTTGGGTATTTG
PPO antisense GGCAATCCGGAGAGATGGT
OX1 sense GCCTGCCAGCCTGTTAGTG
OX1 antisense CAAGGCATGGCCGAAGAG
OX2 sense GAAAGAATATGAGTGGGTCCTGATC
OX2 antisense CAGGACGTTCCCGATGAGA
Cyclophilin sense GTGGCAAGATCGAAGTGGAGAAAC,
Cyclophilin antisense TAAAAATCAGGCCTGTGGAATGTG

Quantitative measurements of PPO, OX1, OX2 and cyclophilin cDNA were performed by kinetic PCR using SYBR green I as fluorescent dye (Invitrogen). PCR reactions consisted of 100 ng cDNA, 0.4 µM primers, 10 mMTris-HCl, 50 mM KCl, 3 mM MgCl2, 0.2 mM deoxy-NTPs, and 1.25 U Taq Polymerase (Invitrogen, Argentina) in a final volume of 25 µl. After denaturizing at 95 °C for 5 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturizing at 95 °C for 15 s, annealing at 62 °C for 40 s and extension at 72 °C for 40 s. The accumulating DNA products were monitored by the ABI7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated based on the quality of dissociation curves, generated at the end of the PCR runs by ramping the temperature of the samples from 60 °C to 95 °C, while continuously collecting fluorescence data. Product purity was confirmed by polyacrylamide gel electrophoresis. Each sample was analyzed in duplicate along with specific standards and no template controls to monitor contaminating DNA. The calculations of the initial mRNA copy numbers in each sample were made according to the cycle threshold (Ct) method. The Ct for each sample was calculated at a fluorescence threshold (Rn) using the ABI7500 sequence detection system software with an automatic baseline setting. For all designed primer sets, linearity of real-time RT-PCR signaling was determined with wide-range serial dilutions of reference cDNA that covered the amount of target mRNA expected in the experimental samples, and clear linear correlations were found between the amount of cDNA and the Ct for the duration of at least 40 real-time RT-PCR rounds.

For the target gene, the relative gene expression was normalized to that of the cyclophilin housekeeping gene by use of the standard curve method, as described by the manufacturer (User bulletin # 2). Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to a sample of 12:00 h of the control group (calibrator sample).

2.6. Western blot analysis

Membrane preparation. Anterior and medial basal hypothalamus, frontoparietal cortex and anterior pituitary were rapidly removed and frozen. The membrane fraction was isolated according to the method of Olpe et al. [41]. Briefly, tissues were homogenized in 10 vols of ice-cold 0.32 M sucrose, containing 1 mM MgCl2 and 1 mM K2HPO4. Homogenates were centrifuged at 750*g*, the supernatant was kept and the pellet was resuspended and the centrifugation repeated. The supernatants were pooled and centrifuged at 18000*g* for 15 min. The pellet was osmotically shocked, centrifuged at 39000*g*, resuspended in 50 mM Tris–HCl, 2.5 mM CaCl2, pH 7.4 (10 vol/g of original tissue), and washed twice. Membranes were frozen at −70 °C.

SDS-10% polyacrylamide gel electrophoresis was then carried out on 50 mg of each of the membrane preparations. Proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were incubated with the polyclonal rabbit antibody (OX1R11-A, Alpha Diagnostics, 1:1000) or alpha syntaxin (1:1000 for hypophysis and 1:4000 for all other tissues, Sigma, MO) at 4 °C followed with a peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz, 1:4000, 1 h at RT), as previously described [26]. Immunoreactive bands were detected using the Western Blotting Chemiluminiscence Luminol Reagent (Santa Cruz Biotechnology). Immunoblots were scanned and quantification was performed with Scion-Image® software (NIH).

2.7. Statistics

Data are presented as mean ± SEM. Differences between treatments were estimated by two-way analysis of variance (ANOVA) followed by Tukey's test for Unequal N post-test using the Statistica Software. $P < 0.05$ indicated statistically significant differences.

3. Results

3.1. Adult control and masculinized females

Testosterone administration to newborn female rats modified serum hormones and the orexinergic system in hypothalamus and pituitary.

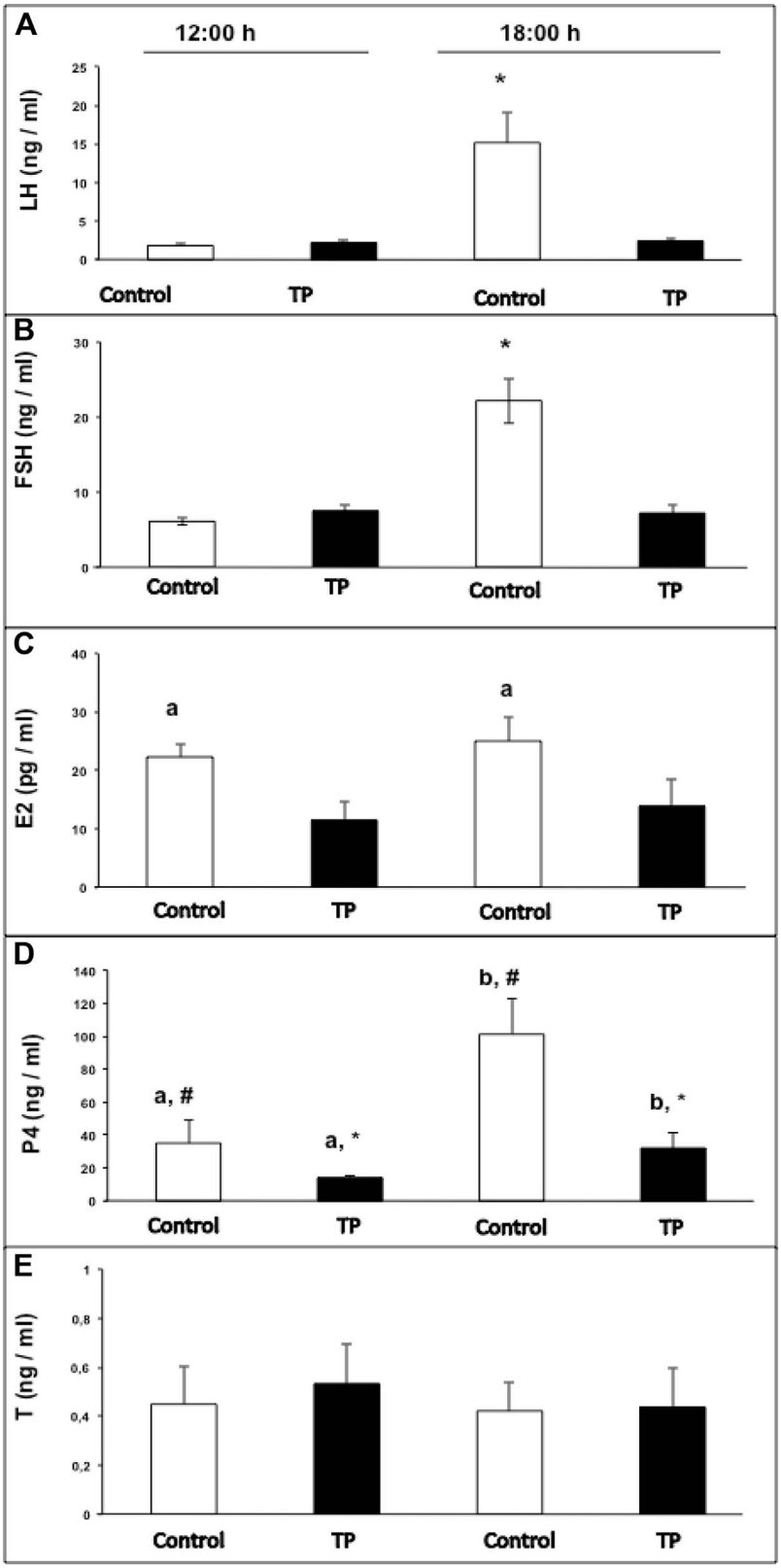

Fig. 1. Hormonal milieu in control proestrous rats (Cont) and in adult androgenized females (neonatally injected with testosterone propionate: TP). N = 4–6.For this and the following figures, all animals were sacrificed at 12:00 and 18:00 h.A: LH (ng/ml) at 12:00 and 18:00. Two way ANOVA: interaction: $p < 0.001$, *: significantly different from all, $p < 0.001$.B: FSH (ng/ml) at noon and 18:00. Two way ANOVA: interaction: $p < 0.03$, *: significantly from Cont at 12:00 h, $p < 0.04$.C: E_2 (pg/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, a: different from TP.D: P_4 (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: a vs b: $p < 0.002$, main effect treatment: # vs *: $p < 0.002$.E: T (ng/ml) at noon and 18:00. Two way ANOVA: NS.

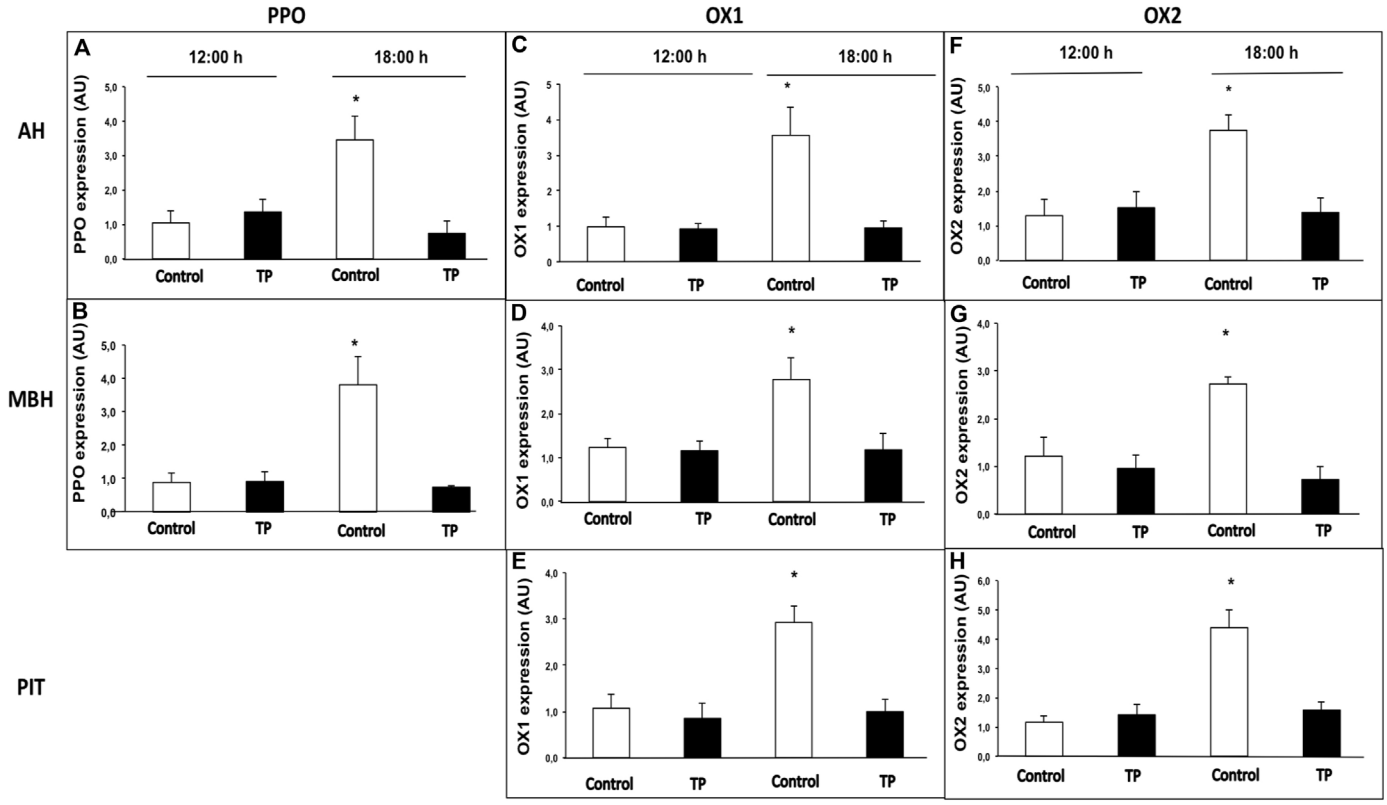

Fig. 2. PPO, OX1 and OX2 mRNA expression in anterior hypothalamus, medial basal hypothalamus and anterior pituitary in control proestrous rats (Cont) and in neonatally androgenized adult females (TP). N = 4–6.A: PPO mRNA expression in anterior hypothalamus (AH). Two way ANOVA: interaction: $p < 0.005$, *: significantly different from all, $p < 0.03$ or less.B: PPO mRNA expression in medial basal hypothalamus (MBH). Two way ANOVA: interaction: $p < 0.03$, *: significantly different from all, $p < 0.05$ or less.C: OX1 mRNA expression in AH. Two way ANOVA: interaction: $p < 0.02$, *: significantly different from all, $p < 0.02$ or less.D: OX1 mRNA expression in MBH. Two way ANOVA: interaction: $p < 0.05$, *: significantly different from all, $p < 0.05$ or less.E: OX1 mRNA expression in pituitary (PIT). Two way ANOVA: interaction: $p < 0.02$, *: significantly different from all, $p < 0.005$ or less.F: OX2 mRNA expression in AH. Two way ANOVA: interaction: $p < 0.02$, *: significantly different from all, $p < 0.03$ or less.G: OX2 mRNA expression in MBH. Two way ANOVA: interaction: $p < 0.02$, *: significantly different from all, $p < 0.03$ or less.H: OX2 mRNA expression in PIT. Two way ANOVA: interaction: $p < 0.002$, *: significantly different from all, $p < 0.05$ or less.

3.1.1. Serum hormones on proestrus

As expected, LH and FSH surged during proestrous afternoon in control adults females but levels of both gonadotropins in neonatally androgenized females were low and did not vary between the selected times (Fig. 1A and B). E_2 was lower in androgenized animals at 12:00 h and 18:00 h (Fig. 1C). P_4 was lower in androgenized females at both times tested; in addition there was an increase in P_4 in the afternoon in both female groups (Fig. 1D). T levels did not differ between treatments and time of day (Fig. 1E).

3.1.2. Expression of PPO, OX1 and OX2 mRNAs in AH, MBH, cortex and pituitary of control and androgenized females at 12:00 and 18:00 h of proestrus

In both anterior hypothalamus (Fig. 2A) and mediobasal hypothalamus (Fig. 2B) a clear increase in PPO expression was present only in normal rats at 18:00 h, at the same time that the gonadotropin surges were detected. PPO was not evaluated in cortex (Ctx) or pituitary (Pit), as it is not expressed in these regions [21,22,24].

Likewise, expressions of OX1mRNA in AH (Fig. 2C), MBH (Fig. 2D) and Pit (Fig. 2E) were significantly increased in control animals at 18:00 h. These increases were absent in neonatally androgenized adult females. The same pattern of expression was observed for OX2 (Fig. 2F–H). In cortex, evaluated as a control tissue, both receptors OX1 and OX2 were expressed, without differences due to treatment or time of day (not shown).

3.2. Adult control and orchidectomized males, treated with E_2-P_4

Next we evaluated whether adult male castration together with a steroid hormone environment similar to the one observed in proestrous females modified hormonal patterns and PPO, OX1 and OX2 expression with regards to normal adult male rats (sham operated). Males were castrated two weeks before the experiment and injected with E_2 or vehicle (at 09:00 h two days before sacrifice) and P_4 or vehicle (at 09:00 h on the day of sacrifice). These treatments did not modify PPO, OX1 and OX2 male expression patterns.

3.2.1. Serum hormones

At 12:00 h, LH values in castrated males, pretreated with oil or steroids, were higher than in their respective controls (Fig. 3A); in addition, at 12:00 h LH levels in castrated-E_2P_4-treated males were significantly lower than in castrated males ($p < 0.05$), demonstrating the negative feed-back effect of E_2 on high LH levels. At 18:00 h, high LH levels were observed in both castrated and steroid-treated-castrated males, without differences between them. In castrated males, LH at 12:00 h was similar to LH at 18:00 h. In contrast, in steroid-treated-castrated males, a significant increase in LH levels was observed at 18:00 h, indicating the effectiveness of steroids treatment to induce LH release ($p < 0.04$).

Serum FSH in castrated males, pretreated with oil or steroids, were higher than the respective controls both at 12:00 and 18:00 h, without differences between them (Fig. 3B). In contrast, steroid treatment significantly decreased FSH levels in control males ($p < 0.01$).

Non-steroid treated castrated males showed significantly lower E_2 and P_4 compared with all the other groups (Fig. 3C-D), demonstrating testis is a source of circulating E_2 and P_4. At both times, higher serum E_2 was present in steroid-injected groups, as expected (Fig. 3C). Furthermore, P_4 injection only significantly increased serum P_4 levels in

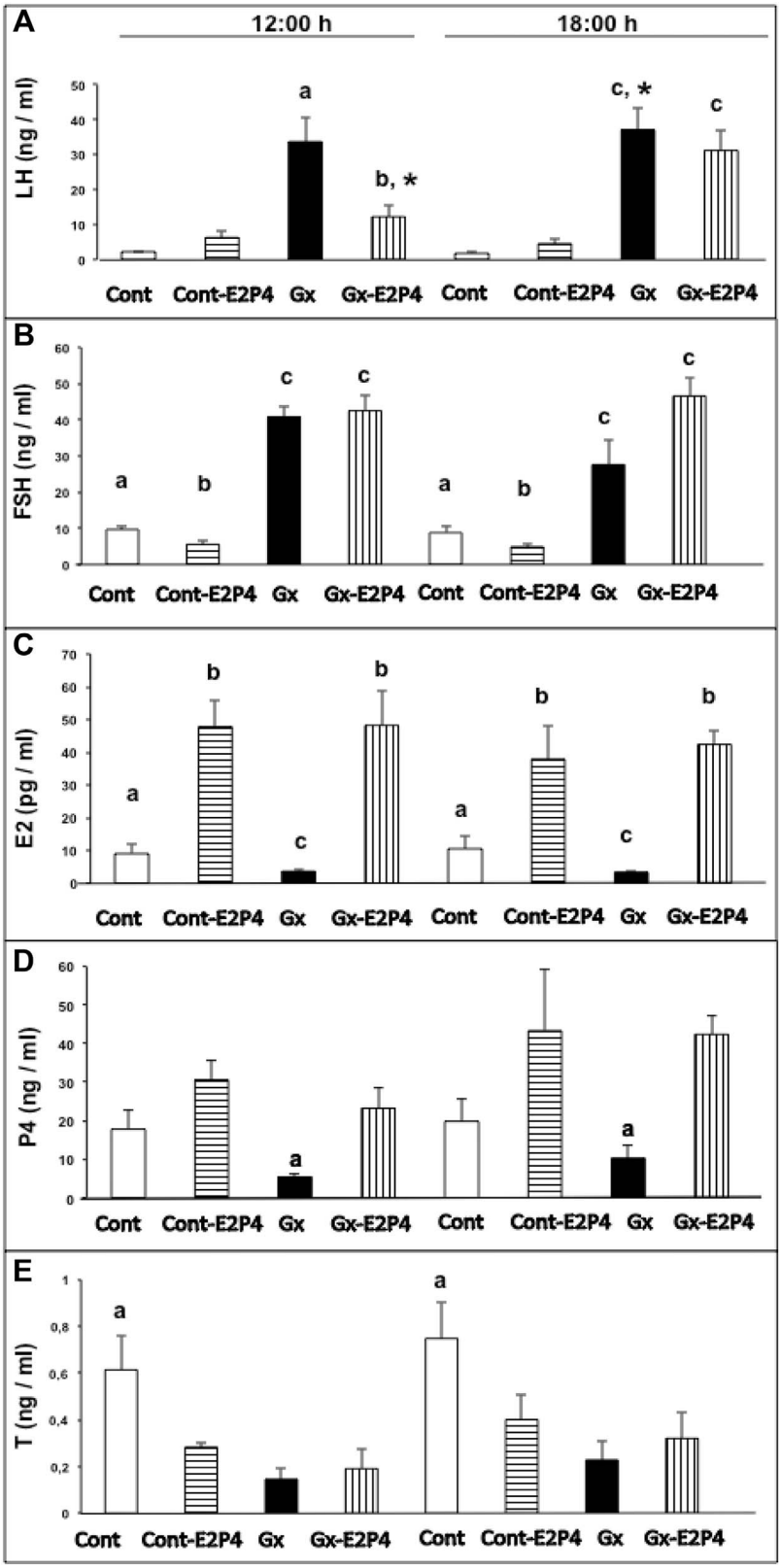

Fig. 3. Hormonal milieu in control male rats and in males orchidectomized in adulthood. A group of males was orchidectomized two weeks before sacrifice (Gx). Both control (Cont) and orchidectomized males were injected with E_2 and P_4 (Cont-E_2P_4; Gx-E_2P_4) or vehicle (Cont; Gx). N = 5–9.A: LH (ng/ml) at 12:00 and 18:00 h. Two way ANOVA: interaction: $p < 0.03$, a: significantly different from all at 12:00 h, $p < 0.05$ or less; b: significantly different from Cont and Gx males at 12:00 h, $p < 0.05$ or less; c: significantly different from Cont and Cont-E_2P_4 at 18:00 h, $p < 0.001$; *: significantly different from Gx-E_2P_4 males at 18:00 h, $p < 0.04$.B: FSH (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS; main effect treatment: $p < 0.001$, a: different from all other treatments, $p < 0.01$, b: different from all other treatments, $p < 0.01$, c: different from Cont and Cont-E_2P_4, $p < 0.01$.C: E_2 (pg/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS; main effect treatment: $p < 0.001$, a: different from all other treatments, $p < 0.01$; b: different from Cont and Cont-E_2P_4, $p < 0.01$, c: different from all, $p < 0.01$.D: P_4 (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS; main effect treatment, $p < 0.001$, a: different from all, $p < 0.001$.E: T (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS; main effect treatment, $p < 0.001$, a: significantly different from Gx and Gx-E_2P_4, $p < 0.01$.

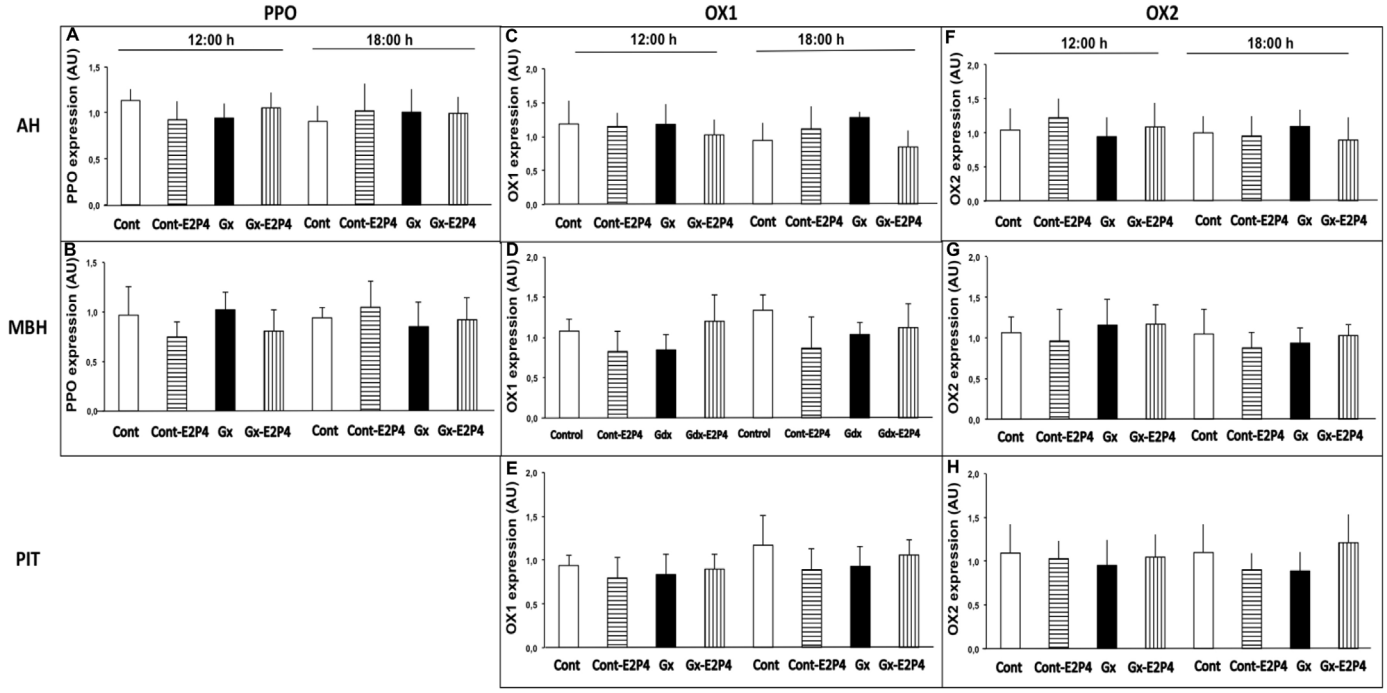

Fig. 4. PPO, OX1 and OX2 mRNA expression in anterior hypothalamus, medial basal hypothalamus and anterior pituitary in control male rats and males orchidectomized in adulthood. Both control and orchidectomized males were injected with E_2 and P_4 (Cont-E_2P_4; Gx-E_2P_4) or vehicle (Cont; Gx). N = 5–9. In all cases (A–H): two-way ANOVA: NS.

castrated males (Fig. 3D). T was lower in all orchidectomized rats independent of steroid treatment (Fig. 3E).

3.2.2. Expression of PPO, OX1 and OX2 mRNAs in AH, MBH, ctx and pit of adult, control and orchidectomized males

Although there were substantial hormonal differences between orchidectomized and control males (with or without steroid treatment) we did not detect any variations in the expression of PPO, OX1 and OX2 mRNAs in either hypothalamic areas or in the adenohypophysis (Fig. 4A–H) or Ctx (not shown), demonstrating that a hormonal environment similar to a proestrous female in the presence of a masculine brain does not modify the expression of the components of the orexinergic system.

3.3. Adult control and demasculinized males

As stated above, altering the endocrine milieu in adult males to a proestrous female-like pattern did not affect the expression of PPO, OX1 or OX2. We next evaluated whether demasculinized males (by injecting with flutamide the dams during pregnancy and thereafter their male pups until day 9, in addition to their orchidectomy on day 11, thus completely hampering the masculinization of the brain) would show an altered expression of our target genes.

Clear results indicate the effect of perinatal demasculinization on both serum hormones and the orexinergic system in AH, MBH and Pit.

3.3.1. Serum hormone levels

Control males showed similar low LH levels at both times tested (Fig. 5A). Demasculinized adult males showed high LH titers at 12:00 h without differences due to E_2P_4 treatment. At 18:00 h serum LH increased in demasculinized males, with or without steroid treatments, with regard to noon, indicating a female-like type of response. In contrast, FSH was increased in both Flut-Gx and Flut-Gx-E_2P_4 groups compared to intact controls at both times of day (Fig. 5B); in all groups levels a 18 h were here higher than at noon ($p < 0.02$), indicating a circadian pattern.

Serum E_2 was higher in groups pretreated with ovarian steroids at both studied times, as expected (Fig. 5C). Flut-Gx males showed decreased P_4 levels compared to controls (Fig. 5D), while P_4 was increased in Flut-Gx-E_2P_4-treated males, as expected. Interestingly, P_4 was higher in the afternoon than at 12:00 h in all groups ($p < 0.05$).

T was significantly lower in both demasculinized groups compared to controls (Fig. 5E).

3.3.2. PPO, OX1 and OX2 expression in AH, MBH, ctx and pit of adult control and demasculinized males

Adult demasculinized males showed high PPO expression at 12:00 h and 18:00 h in both hypothalamic areas only when treated with E_2P_4 (Fig. 6A–B). In contrast, in adult, control and castrated males E_2P_4 treatment had not modified the expression of any of the components of the orexinergic system, as shown in Fig. 4.

Likewise, adult demasculinized males showed high OX1 and OX2 mRNA expression at 12:00 h and 18:00 h in both hypothalamic areas and the pituitary only when injected with E_2-P_4 (Fig. 6C–H). These differences were not observed in cortex, where levels remained constant despite treatments (not shown).

In Flut-Gx-E_2P_4 males we observed no differences in PPO, OX1 and OX2 expression between 1200 and 1800 h in any tissue.

3.4. Western blot analysis of OX1 protein expression in AH, MBH, Ctx and pit in control and masculinized females and control and demasculinized males

To determine whether the changes observed in OX1 gene expression were also apparent at the protein level, the molecule with physiologic relevance, we performed western blot analysis in the groups where changes at the gene level were observed, i.e. control and masculinized females and control and demasculinized males.

A significant increase of OX1 content was observed in both hypothalamic areas and the pituitary (Fig. 7A–C), but not in Ctx (not shown) at 1800 h with regard to 12:00 h in proestrous rats, but not in androgenized females, correlating with mRNA expression of this gene.

In contrast, in demasculinized-E_2P_4-treated males significant increases in OX1 expression in both hypothalamic areas and pituitary (Fig. 7D–F), but not in Ctx (not shown), were observed at both 12:00 and 18:00 h, again correlating with mRNA expression.

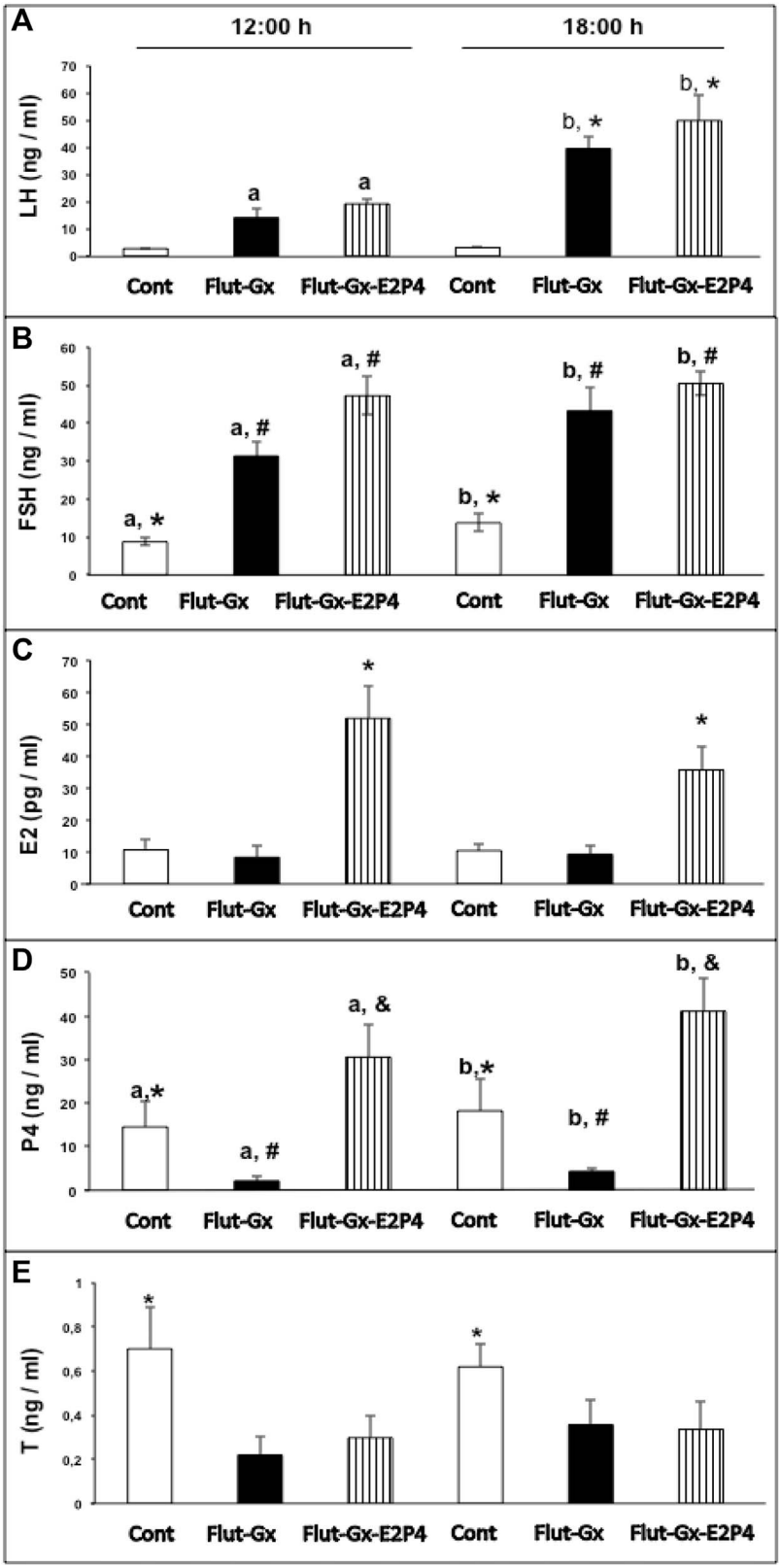

Fig. 5. Hormonal milieu in control male rats (Cont) and in neonatally demasculinized males (treated with flutamide and later orchidectomized). A group of demasculinized males were injected with E_2 and P_4 (Flut-Gx-E_2P_4) or vehicle (Flut-Gx). N = 4–6.A: LH (ng/ml) at 12:00 and 18:00 h. Two way ANOVA: interaction: $p < 0.02$, a: significantly different from Cont at 12:00 h, $p < 0.01$; b: significantly different from Cont at 18:00 h, $p < 0.01$; *: significantly different from 12:00 h for each treatment, $p < 0.01$.B: FSH (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: a (12:00 h) different from b (18:00 h), $p < 0.02$; main effect treatment: # different from *, $p < 0.001$,C: E_2 (pg/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS; main effect treatment: $p < 0.01$, *: different from Controls and Flut-Gx, $p < 0.01$.D: P_4 (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: a (12:00 h) different from b (18:00 h), $p < 0.05$; main effect treatment, $p < 0.001$, *: different from Flut-Gx and Flut-Gx-E_2P_4, $p < 0.03$ or less, #: different from Cont and Flut-Gx-E_2P_4, $p < 0.01$, & : different from Cont and Flut-Gx, $p < 0.03$ or less.E: T (ng/ml) at noon and 18:00. Two way ANOVA: interaction: NS, main effect time: NS; main effect treatment, $p < 0.001$, *: significantly different from Flut-Gx and Flut-Gx-E_2P_4, $p < 0.04$ or less.

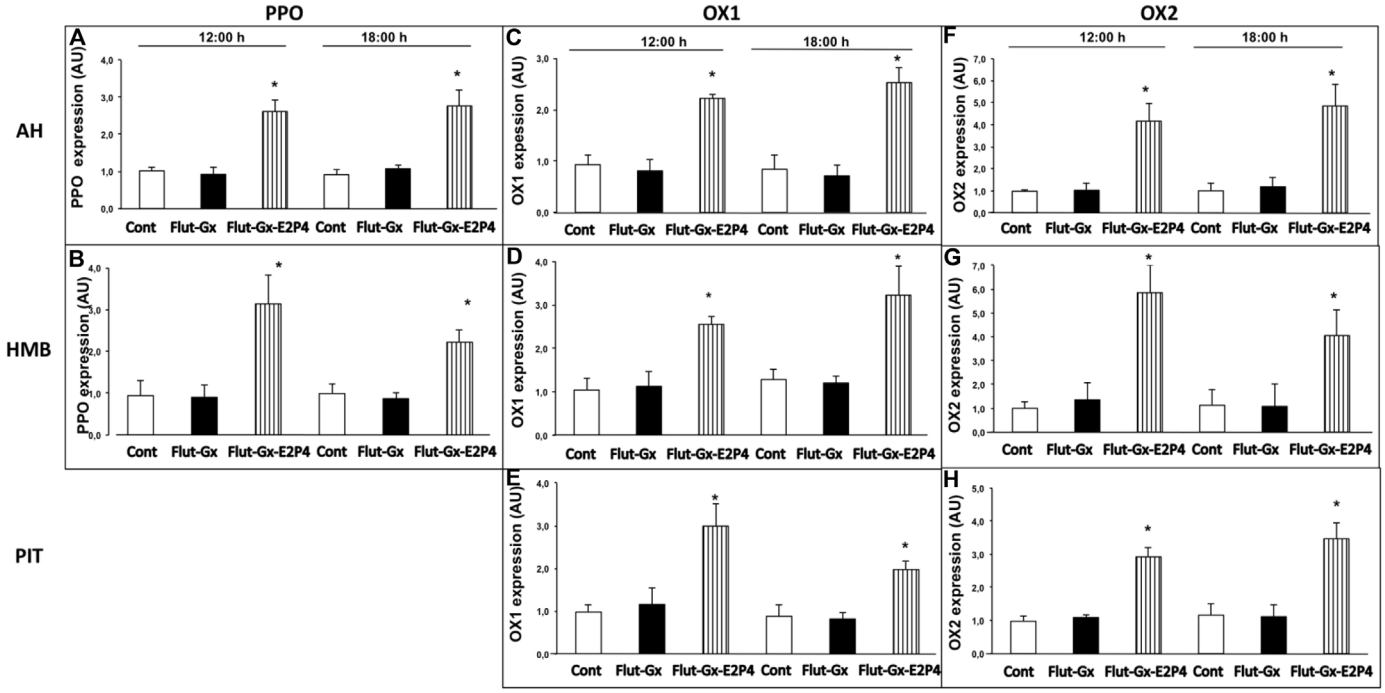

Fig. 6. PPO, OX1 and OX2 mRNA expression in anterior hypothalamus, medial basal hypothalamus and anterior pituitary in control male rats (Cont) and in males neonatally demasculinized (treated with Flut and later orchidectomized). A group of demasculinized males were injected with E_2 and P_4 (Flut-Gx-E_2P_4) or vehicle (Flut-Gx). N = 4–6.A: PPO mRNA expression in anterior hypothalamus (AH). Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment, $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.01$.B: PPO mRNA expression in medial basal hypothalamus (MBH). Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.01$.C: OX1 mRNA expression in AH. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.01$.D: OX1 mRNA expression in MBH. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.02$, *: significantly different from Cont and Flut-Gx, $p < 0.04$.E: OX1 mRNA expression in pituitary (PIT). Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.02$.F: OX2 mRNA expression in AH. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.01$.G: OX2 mRNA expression in MBH. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.01$.H: OX2 mRNA expression in PIT. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different from Cont and Flut-Gx, $p < 0.01$.

4. Discussion

The orexinergic system has been postulated to participate in the brain control of reproduction [14–23]. In addition, sex differences in PPO and OX1 expression levels were reported in hypothalamus (PPO: females > males) [22] and pituitary (OX1: males > females) [21]. We have previously proposed the participation of the orexinergic system in the neuroendocrine events necessary for attaining successful reproduction in females. In adult cycling female rats the expression of PPO, OX1 and OX2 peaked during the evening of proestrus selectively in hypothalamus and adenohypophysis. Since no changes were observed in males in any region at any time, those changes suggested that they are sex specific, cycle-related events associated to this particular hormonal status in the female [24–27].

During the process of sexual differentiation, genes can be permanently turned on or shut off to acquire sex specific expression patterns; alternatively, gene expression may transiently be sexually differentiated, inducing or preventing sex-specific responses at a particular time, and then lose this sexual-specific expression to attain similar expression levels in adulthood [30,33,42]. The gestational and perinatal environments are important periods for neural programming mechanisms. Since connections linking reproduction and energy balance develop in utero [28,43], here we studied in rodents the effect of the presence or absence of testosterone in the critical period of brain sexual differentiation on the dimorphic sexual expression of the orexinergic system observed previously in adult animals.

In a first set of experiments, newborn female rats were injected with testosterone to masculinize the brain control of gonadotropins secretion, i.e., suppress FSH and LH surges, estrous cyclicity and ovulation. The neonatal steroid treatment effectively suppressed gonadotropic peaks on proestrous afternoon, as expected. Also, E_2 and P_4, but not T, were lower in these infertile animals. A clear, specific activation of the orexinergic system in hypothalamus and pituitary was observed in normal females only during the afternoon, but not in androgenized rats. Thus, lack of the organization of a female brain is followed by absence of gonadotropic peaks and lack of activation of PPO expression in hypothalamus and lack of activation of the expression of both OX1 and OX2 receptors in hypothalamus and pituitary.

In demasculinized males, due to perinatal flutamide injections and orchidectomy, increases of LH and FSH are present in the afternoon with or without steroid treatment, indicating that in the demasculinized male brain gonadotropin secretion is coordinated with the time of day. Interestingly, only demasculinized males pretreated with E_2 and P_4 showed a clear activation of the orexinergic system in comparison to controls, similar to what we observed in normal proestrous females [24–26], though in this case increases in PPO, OX1 and OX2 expression were observed both at noon and in the late afternoon.

These results suggest that additional feminization of the female brain is also required to obtain a full female response, as also proposed for other systems [44].

In agreement with the concept that perinatal testosterone differentiates the brain towards a male organization, males castrated in adulthood showed the typical increases in gonadotropins, but the expression of PPO, OX1 and OX2 mRNAs was not modified in any of the tissues analyzed. These results differ from those obtained by Jöhren et al. in pituitary OX1 mRNA expression in castrated males [45], as they observed a significant increase due to castration. Differences in several experimental conditions may account for this discrepancy (castration duration, animal strain, etc.).

The fact that neonatal testosterone induces the sex specific differ-

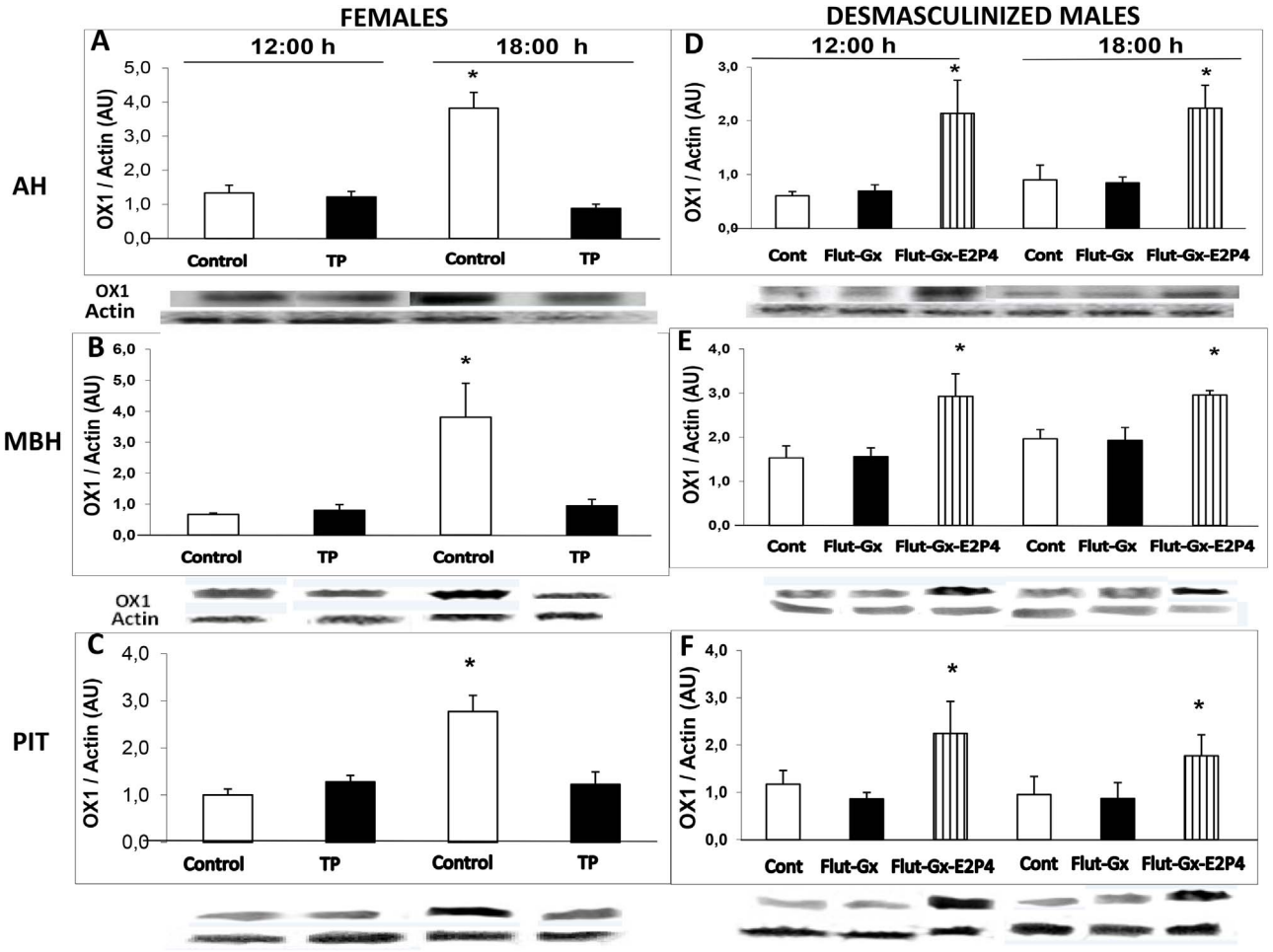

Fig. 7. OX1 protein expression in anterior hypothalamus, medial basal hypothalamus and anterior pituitary in control (Cont) and androgenized female rats (TP) and in control (Cont) and neonatally demasculinized males (treated with flutamide and later orchidectomized). A group of demasculinized males were injected with E_2 and P_4 (Flut-Gx-E_2P_4) or vehicle (Flut-Gx). N: females = 4–6, males = 4–6. In all panels under the bar graph there is a representative Western blot of the protein analyzed.A: OX1 protein expression in AH in females. Two way ANOVA: interaction: $p < 0.05$, *: significantly different from all, $p < 0.05$ or less.B: OX1 protein expression in MBH in females. Two way ANOVA: interaction: $p < 0.02$, *: significantly different from all, $p < 0.01$ or less.C: OX1 protein expression in pituitary (PIT) in females. Two way ANOVA: interaction: $p < 0.001$, *: significantly different from all, $p < 0.001$ or less.D: OX1 protein expression in AH in males. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.001$, *: significantly different Cont and Flut-Gx, $p < 0.001$.E: OX1 protein expression in MBH in males. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.001$, *: significantly different Cont and Flut-Gx, $p < 0.001$.F: OX1 protein expression in pituitary (PIT) in males. Two way ANOVA: interaction: NS, main effect time: NS, main effect treatment: $p < 0.01$, *: significantly different Cont and Flut-Gx, $p < 0.05$ or less.

entiation of the brain control of gonadotropin secretion in parallel to the sex specific expression of the orexinergic system, and that we and others have shown that orexins stimulate LH secretion from pituitary cell cultures from different experimental models [27,46], suggests that the increases in PPO, OX1 and OX2 expression at the time of the preovulatory gonadotropin peaks may form part of a synchronized complex of inputs that ensures the proper secretion of these vital hormones for reproduction. In agreement with this hypothesis, we have shown that blocking orexin actions on proestrus by injecting orexin receptor antagonists decreased the preovulatory gonadotropin surges and reduced ovulation [25].

Interestingly, orexins have also been proposed to facilitate sexual behavior, adding another important aspect to the synchronicity of both systems. The role of orexin neurons in the activation of different aspects of sexual behavior in male rats has been extensively studied [47], nevertheless, almost no literature is available regarding female sexual behavoir. It is therefore possible to postulate that the female-specific late proestrous increases of hypothalamic PPO and orexin receptors expression, at the time of sexual receptivity, may participate in modulating some aspects of this behavior, critical for mating. Future studies will evaluate this hypothesis.

5. Conclusions

The sex specific development of the neuroendocrine control of gonadotropin secretion in coordination with the sex specific development of the hypothalamic-pituitary orexinergic system in response to perinatal sex hormones suggests an interrelationship between both systems in the control of reproduction. These results are an example of gestational/neonatal programming: a specific challenge (testosterone) during a critical developmental time (perinatally) modifies brain organization, with persistent effects on the offspring phenotype (synchronic brain masculinization of the gonadotropin and the orexinergic systems) [48,49].

Acknowledgements

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