Gonadal steroids modulated hypocretin/orexin type-1 receptor expression in a brain region, sex and daytime specific manner

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\textbf{A B S T R A C T}

Orexins A and B (hypocretins A and B) are regulatory peptides that control a variety of neuroendocrine and autonomic functions including feeding and sleep-wakefulness. Previously, we described a clear relationship between the hormonal milieu of the estrous cycle and the mRNA expression of the components of the orexigenic system, in the hypothalamus, pituitary and ovary. Here, we investigate whether steroid hormones are involved in the modulation of the hypocretin/orexin type-1 receptor expression at the protein level, and its time of the day dependence, in hypothalamus and pituitary of castrated male and female rats and castrated receiving hormone replacement. Orchidectomy decreased the hypocretin/orexin type-1 receptor expression in anterior hypothalamus, but not in mediobasal hypothalamus or cortex; in pituitary this treatment resulted in an increase. Testosterone and dihydrotestosterone were able to restore receptor expression and gonadotropins. In females, pituitary and ovarian hormones increased during proestrous afternoon. Hypocretin/orexin type-1 receptor expression was higher at 19:00 of proestrus in hypothalamus and pituitary. Ovariectomized treated with estradiol or oil and sacrificed at 11:00 h showed the receptor expression similar to 11:00 h of proestrus in hypothalamus and pituitary. At 19:00 h, low expression persisted in these areas in oil-treated ovariectomized rats; in contrast, estradiol replacement increased the expression to high levels of normal cycling rats at 19:00 h. Sexual steroids modulate the orexigenic system and the anatomical regions, hormones and times of the day all have to be considered when the roles of orexins, and probably other peptides, are under consideration.

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1. Introduction

Orexins A and B (also known as hypocretins A and B) are regulatory peptides that control a variety of neuroendocrine and autonomic functions including feeding and sleep-wakefulness. They act via their hypocretin/orexin type-1 (OX1) and hypocretin/orexin type-2 (OX2) receptors [1–7]. Both neuropeptides are potent agonists at both OX1 and OX2 G protein-coupled receptors. Previous studies suggested a role of OX1 receptor in food intake, metabolism and reproduction [8,9]; whereas OX2 receptor was shown to play a role in sleep-wakefulness regulation [10]. Orexins have been implicated in multiple functional pathways including the control of GnRH neurons and the secretion of pituitary gonadotropins, in a variety of \textit{in vivo} and \textit{in vitro} experiments, in rodents [11–19] and humans [20].

It has been reported that intracerebroventricular administration of orexin A stimulates LH secretion in castrated female rats primed with estradiol and progesterone and inhibits LH secretion in unprimed rats. This dual effect may be due to a steroid regulation of the orexin receptors [15].

On the other hand, classic experiments have shown that ovulation and estrous cyclicity are under circadian control [21]. In a variety of rodents, a role for the circadian clock in the regulation of the timing of the preovulatory LH surge has been demonstrated [22]. In a previous work we described a particular relationship between the hormonal milieu of the estrus cycle and the mRNA expression of the components of the orexigenic system in the hypothalamus, pituitary and ovary. We demonstrated a significant increase in these gene expressions in the afternoon and night of proestrus that correlated with hormonal levels. In addition, we demonstrated an important role for gonadotropins as regulators of the OX1 and OX2 receptors expression [9,17]. Here, we investigate whether steroid hormones are involved in the modulation of the OX1 receptor expression at the protein level, and its time of the day dependence, in hypothalamus and pituitary of castrated male and female rats and animals receiving hormone replacement therapy.
2. Materials and methods

2.1. Animals

Adult male and female Sprague-Dawley rats (200–250 g) 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. All studies on animals were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) and by the NIH.

Males were castrated or sham castrated (control males) under light ether anesthesia. Orchidectomized males (MOR) were injected 6 times with either castor oil (vehicle control) or with Testosterone Propionate at the following doses: 150 μg (TP150) or 300 μg (TP300), or with dihydrotestosterone (DHT) at the following doses: 250 μg (DHT250) or 500 μg (DHT500), during two weeks. Rats were killed by decapitation two weeks after castration, in the morning (9:00–10:00 AM). Serum, anterior hypophysis, anterior and mediobasal hypothalamic [9,17] and frontoparietal cortex were collected and frozen at −70 °C.

A group of cycling females was castrated (FOVFOV) under light ether anesthesia and received 6 doses of either vehicle (castor oil) or 10 μg estradiol benzoate (EB). Regular cycles were defined as the occurrence of 3 consecutive 4 day cycles. A group of cycling females on proestrus was kept as controls and these females were sacrificed at 11:00 AM (P11) or 19:00 AM (P19). The same tissues were collected and frozen at −70 °C.

2.2. Drugs

Testosterone Propionate, Dihydrotestosterone (5α-androstan-17β-ol-3-one) and Estradiol Benzoate were acquired from SIGMA-ALDRICH, St. Louis, MO.

2.3. Membrane preparation and Western blot analysis

Anterior and mediobasal hypothalamus, frontoparietal cortex and ovaries from rats were rapidly removed and frozen. The membrane fraction was isolated according to the method of Olpe et al. [23]. Briefly, tissues were homogenized in 10 volumes 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 18,000 × g for 15 min. The pellet was osmotically shocked, centrifuged at 39,000 × 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 μg 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). Immunoreactive bands were detected using the Western blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology). Immunoblots were scanned and quantification was performed with ScionImage® software (NIH).

To test the specificity of the OX1 antibody, a Western blot using tissues previously reported to show or lack this receptor expression at the mRNA level [24,25] was carried out. A 47.5 kDa band corresponding to the OX1 receptor was present in the positive controls (MBH and kidney), whereas no bands were detected in the lung (negative control), confirming the antibody’s tissue specificity. Beta actin (Calbiochem, 1:3000, 2 h at RT) was used as a loading control, showing a 43 kDa band (Fig. 1).

2.4. Hormone determinations

Serum LH, FSH and PRL were determined by RIA using kits obtained through NHPP, NIDDK and Dr. A. Parlow. Results were expressed in terms of RP3 (reference preparation 3) rat LH, FSH and PRL standards. Assay sensitivities were 0.015 ng/ml for LH, 0.1175 ng/ml for FSH and 1.6 ng/ml for PRL. Intra- and inter-assay coefficients of variation for LH were 7.2% and 11.4%, respectively, for FSH 8.0% and 13.2%, respectively and 8.1% and 11.4%, respectively, for PRL.

Serum estradiol, progesterone and testosterone were determined by RIA using specific antiserum kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO, USA) after ethyl ether extraction. Labeled estradiol and progesterone hormones were purchased from Perkin Elmer (Wellesley, MA, USA). Assay sensitivities were 11.3 pg for estradiol and 500 pg for progesterone. Intra-and inter-assay coefficients of variation were 6.8 and 11.7% for estradiol, respectively; 7.1 and 12.15% for progesterone, respectively. Labeled testosterone was purchased from New England Nuclear. Assay sensitivity for testosterone was 12.5 pg and intra- and inter-assay coefficients of variation were 7.8% and 12.3% respectively.

2.5. Statistics

Data are presented as mean ± SEM. Differences between treatment groups were estimated by one-way analysis of variance (ANOVA) followed by Tukey’s post-test using the Statistica Software. Frequency distributions were analyzed using the $\chi^2$ test. $p < 0.05$ indicated statistically significant differences.

3. Results

3.1. Serum hormones

3.1.1. Males

Both androgen treatments effectively reversed the effects of orchidectomy on gonadotropin levels in a dose-dependent manner (Fig. 2). Both TP and DHT significantly increased circulating testosterone in MOR rats, though not attaining normal male levels.

3.1.2. Females

Estradiol, progesterone, prolactin and gonadotropins levels in controls revealed the proestrous peaks at 19:00 h previously described for our colony [9,17]. Castration increased LH and FSH while significantly decreasing PRL and estradiol with regards to 11:00 h on proestrus (P11). Estradiol treatment effectively restored serum estradiol in both groups of FOV-EB animals (11:00 h and 19:00 h) and lowered circulating LH and FSH levels. Both treatments significantly increased testosterone levels compared to controls (P11 vs. P19) and lowered circulating prolactin levels.

![Fig. 1. OX1 receptor antibody tissue specificity. Western blot for OX1 (upper panel) and beta actin (lower panel) were carried out in mediobasal hypothalamus (MBH), kidney and lung extracts.](image-url)
19:00 h) to values similar to those found during proestrous afternoon (Fig. 3).

3.2. OX1 receptor expression

3.2.1. Males

Immunoblotting experiments using the anti-OX1 antibody (OX1R11-A) detected a specific band (47.5 kDa, equivalent to the native OX1 receptor) in agreement with previous data [22], in the hypothalamus, pituitary and frontoparietal cortex. Orchidectomy significantly decreased the relative expression in anterior hypothalamus (AH) but not in mediobasal hypothalamus (MBH) or frontoparietal cortex (FC). However, in pituitary (P), MOR resulted in a three-fold increase of OX1 expression (Fig. 4). Androgen treatments were able to restore MOR effects in AH and P.

3.2.2. Females

Fig. 5 shows OX1 expression measured by Western blots in AH, MBH, P and frontoparietal cortex (FC) as control, from FOV, FOV-EB and proestrous rats sacrificed at 11:00 h on proestrus (P11) or at 19:00 h on proestrus (P19). OX1 expression resulted higher in females sacrificed at 19:00 h of proestrus in hypothalamus and...
pituitary, in agreement with our previous results using Real Time RT-PCR [9,17].

Both FOV and FOV-EB sacrificed at 11:00 h showed OX1 expression similar to 11:00 h of proestrus in AH, MBH and P. At 19:00 h in FOV animals, low OX1 expression persisted in these areas; in contrast, estradiol replacement in FOV-EB animals significantly increased OX1 expression to high levels typical of normal cycling rats at 19:00 h in the mentioned tissues.

In FC, no differences were observed between the treatments and at different times of the day.

4. Discussion

In addition to the original functions attributed to orexins, some evidences from our laboratory [9,17] and also from others [12,14–16,19] suggested the participation of orexins in the regulation of hypothalamus-pituitary-gonadal axis. In previous works we described a particular relationship between the hormonal milieu of proestrus and the expression of components of the orexinergic system at the hypothalamic, pituitary and ovarian levels [9,17].

In a previous work, a screening of different tissues reported a differential expression of OX1 and OX2 receptors in males and females [24], suggesting a gonadal steroid regulation of these receptors' expression. Our results demonstrate that OX1 receptor expression is differentially regulated by testosterone in AH and P in males. In the absence of testosterone, OX1 receptor protein expression decreases in the anterior hypothalamus but increases in the pituitary and both expression levels are restored by hormone replacement. In a previous report [25], no differences in hypothalamic OX1 mRNA expression measured by Real Time PCR in MOR and hormone-replaced animals were reported. Here we demonstrate that OX1 receptor protein expression regulation by testosterone is specific of the anterior hypothalamic area, possibly explaining differences between the current data and the previous study [25]. In addition, differences in testosterone regulation of transcription and translation of OX1 receptor expression cannot be discarded.

In females, the previously reported specific increments of OX1 expression in late proestrus in hypothalamus and pituitary were confirmed by Western blot. FOV animals sacrificed at 11:00 and 19:00 h showed low OX1 receptor levels, similar to those of proestrous at 11:00 h (P11) in AH, MBH and P. However, only FOV-EB females sacrificed at 19:00 h restored the high OX1 receptor expression to levels comparable to proestrous at 19:00 h (P19) in these tissues. These results suggest that OX1 receptor expression in selected areas, as the hypothalamus and the adenohypophysis, is under the influence of estradiol and the time of the day in the adult.

Fig. 4. OX1 receptor expression measured by Western blot in anterior hypothalamus (AH), mediobasal hypothalamus (MBH), pituitary (P) and frontoparietal cortex (FC) of control, castrated (MOR) and MOR replaced with testosterone propionate: 150 µg (MOR TP150), 300 µg (MOR TP300); or dihydrotestosterone: 250 µg (MOR DHT250), 500 µg (MOR DHT500). synt: syntaxin. AU: arbitrary units. (*: different from control, p<0.05, n=6).
female rat. This hypothesis is strengthened whenOX1 receptor expression results are observed together with hormonal levels in control proestrous cycling animals. Estradiol levels peak at 11:00 h and 19:00 h, but OX1 receptor expression is only increased in the afternoon. It is important to mention that estradiol levels are constantly high and similar in both FOV-EB animals sacrificed at 11:00 h and 19:00 h.

Several implications of the present results can be inferred. Previous data from different rodent species suggested the occurrence of estrous cycle-related events in specific times of the day, usually related with the circadian system. Many animal species show seasonal rhythms in physiology and behavior. The processing of light information by the circadian system of mammals has evolved on many levels of organization, such as receptors, pathways, processing neurons and genes [26,27]. Ovulation occurs in a fixed time relative to the light–dark cycle and LH and FSH release are also controlled by the neural circadian system. Suprachiasmatic nucleus neurons comprise the neural clock involved in the circadian signals related to the proestrous hormonal release, ovulation and sexual receptivity; events that occur in specific times of the day [28].

Our present results suggest a combined participation of estradiol and the clock system in the regulation of hypothalamic OX1 expression in late proestrus. Furthermore, previous results from our laboratory using the OX1 receptor selective antagonist SB-334867 or the OX2 receptor selective antagonist JNJ-10397049, alone or combined, in proestrus afternoon, decreased serum gonadotropins and reduced ova number the following (estrus) morning. In SB-334867 treated rats, ovaries showed a bloody reaction with more preovulatory follicles and less corpora lutea [20].

Testosterone and DHT in males, as well as estradiol in females, showed opposite effects on the pituitary regulation of OX1 receptor expression. The sexual dimorphism observed for the androgen and estrogen effects is consistent with previous observations on some metabolic variables [29–31].

In summary, in addition to the well-known regulatory action of the orexins on the reproductive axis, here we demonstrate that sexual steroids also modulate the orexinergic system.

Anatomical regions, sexual dimorphism, hormones and times of the day have to be considered when the role of orexins, and probably other peptides, is under consideration.

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Fig. 5. OX1 receptor expression measured by Western blot in anterior hypothalamus (AH), mediobasal hypothalamus (MBH), pituitary (P) and frontoparietal cortex (FC) of proestrous females sacrificed at 11:00 h (P11) and 19:00 h (P19), ovariectomized and sacrificed at 11:00 h (FOV 11) or 19:00 h (FOV 19) and FOV replaced with 10 μg of estradiol benzoate and sacrificed at 11:00 h (FOV-EB 11) or 19:00 h (FOV-EB 19). (*: different from 11:00 h on proestrus (P11), p<0.05, n = 5).
References


