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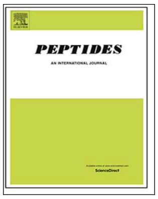

Perinatal programming of the orexinergic (hypocretinergic) system in hypothalamus and anterior pituitary by testosterone

Natalia I. Cataldi[a], Victoria A. Lux-Lantos[a,*], Carlos Libertun[a,b]

[a] *Instituto de Biología y Medicina Experimental-CONICET, Argentina*
[b] *Universidad de Buenos Aires, Facultad de Medicina, Departamento de Fisiología y Biofísica, Buenos Aires, Argentina*

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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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[*] Corresponding author at: Neuroendocrinology Laboratory, IBYME-CONICET, Vuelta de Obligado, 2490, Buenos Aires, Argentina.
E-mail addresses: nataliacataldi@hotmail.com (N.I. Cataldi), vlux@ibyme.conicet.gov.ar, vlux@lantos.com.ar (V.A. Lux-Lantos), libertun@dna.uba.ar (C. Libertun).

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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%,