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Suckling-induced *Fos* activation and melanin-concentrating hormone immunoreactivity during late lactation

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ABSTRACT

Aims: Melanin-concentrating hormone (MCH) is implicated in the control of food intake, body weight regulation and energy homeostasis. Lactation is an important physiological model to study the hypothalamic integration of peripheral sensory signals, such as suckling stimuli and those related to energy balance. MCH can be detected in the medial preoptic area (MPOA), especially around the 19th day of lactation, when this hormone is described as displaying a peak synthesis followed by a decrease after weaning. The physiological significance of this phenomenon is unclear. Therefore, we aimed to investigate hypothalamic changes associated to sensory stimulation by the litter, in special its influence over MCH synthesis.
Main methods: Female Wistar rats ($n = 56$) were euthanized everyday from lactation days 15–21, with or without suckling stimulus (WS and NS groups, respectively). MCH and *Fos* immunoreactivity were evaluated in the MPOA and lateral and incerto-hypothalamic areas (LHA and IHy).
Key findings: Suckling stimulus induced *Fos* synthesis in all regions studied. An increase on the number of suckling-induced *Fos*-ir neurons could be detected in the LHA after the 18th day. Conversely, the amount of MCH decreased in the MPOA from days 15–21, independent of suckling stimulation. No colocalization between MCH and *Fos* could be detected in any region analyzed.
Significance: Suckling stimulus is capable of stimulating hypothalamic regions not linked to maternal behavior, possibly to mediate energy balance aspects of lactation. Although dams are hyperphagic before weaning, this behavioral change does not appear to be mediated by MCH.

1. Introduction

Melanin-concentrating hormone (MCH) is a nonadecapeptide that was first discovered in the fish class Teleostei and later described in mammals [1–4]. In the mammalian brain, MCH is synthesized mainly in cell bodies of hypothalamic sites such as in the lateral hypothalamic area (LHA), perifornical area and, to a lesser degree, in the incerto-hypothalamic area (IHy) [5]. Exclusively during the lactation period, MCH synthesis can be detected in neurons of the medial preoptic area, raising from the 5th to the 19th day and decreasing concomitantly to the weaning of the pups [6, 7]. The exact role played by MCH in physiological alterations of motherhood is still unclear. The projections of MCH-immunoreactive [MCH-ir] neurons are broad, reaching for instance the median eminence, hippocampal formation, prefrontal cortex, periaqueductal gray matter, lateral part of the medial mammillary nucleus, nucleus accumbens, and medial septal nucleus. Thus, LHA and IHy can be considered integrative centers that are optimally positioned to influence a wide range of systems and functions, not simply as direct modulators of effector and motor functions [8]. Melanin-concentrating hormone is involved in the regulation of body weight, acting on G-protein-coupled receptors similarly to other central and peripheral peptides, such as orexin, cholecystokinin, and bombesin [9–11]. Additionally, intra-medial preoptic area injections of MCH altered monoamine concentrations in this area and stimulated sexual behavior and luteinizing hormone secretion, as well as a decrease in appetitive components of maternal behavior of dams [12–14]. The present study evaluated the daily influence of the suckling stimulus on MCH and *Fos* immunoreactivity (*Fos*-ir) in the LHA, MPOA, and IHy during late lactation.

2. Materials and methods

2.1. Animals

Adult Wistar rats ($n = 56$) were mated with approximately 90 days old at the beginning of the experiments. The rats were obtained from the Department of Pathology, School of Veterinary

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Medicine, University of São Paulo, and housed in polypropylene cages (30 cm × 40 cm × 18 cm) with approximately 1.0 l of medium-grade pine flakes as bedding under a 12 h/12 h light/dark cycle (lights on at 6:00 AM) with controlled temperature (23 ± 2 °C). Rat chow and water were available ad libitum throughout the experiment. The animal housing and procedures were in accordance with the guidelines of the Committee on Care and Use of Animal Resources, School of Veterinary Medicine, University of São Paulo (proc. no. 2185/2011). These guidelines are similar to those of the National Research Council, USA.

2.2. Mating

At the end of the light period, female rats that were in the period of transition from proestrus to estrus were placed in the cage of a sexually experienced male. The day that sperm was observed in the vaginal lavage of each female was designated day 1 of pregnancy. On the second day after birth, the litters were culled to eight pups each (four females and four males).

2.3. Experimental design

The eight pups were left with the mother and the lactation progressed without interference until the weaning period, when animals were separated into two groups: by presence or not of suckling stimulus ($n = 28$ for each group — NS, no suckling — WS, normally exposed to suckling pups). Among those groups quoted above, again the animals were divided into subgroups, now by the day of sacrifice ($n = 4$ for each subgroup, again NS and WS) on the 15th to 21st day of lactation. To create the NS group, we employed the vibrissae pad anesthesia technique employed by other authors [15, 16]. Pups were separated from the mother 1 h before the test, and only in the NS group the pups received a topic cream of 5% lidocaine (Xylocaína 50 mg/g — Astra Zeneca; London, United Kingdom) in their vibrissae pads, resulting in complete loss of vibrissal reflexes. They were returned to the mother and their capability to attach to the nipples was assessed. In cases where pups in the NS group were able to attach to the nipples, the mothers have been discontinued from the experiment. In both groups there was no impairment in the pups' capability to vocalize or in the mother retrieval, grooming and nest building. After 20 min, pups were again separated from the mother and after 90 min — time to allow *Fos* synthesis — the dams were perfused. Tissue was then subjected to double immunohistochemical staining to identify regions of *Fos*- and MCH-ir. (See Fig 1.)

2.4. Immunohistochemistry for double-labeling hypothalamic neurons with MCH and Fos

All of the animals ($n = 56$) that were used in the experiments above were anesthetized with a lethal dose of 35% chloral hydrate (1 ml, i.p.) and perfused via the ascending aorta first with cold 0.9% saline (100 ml for ~1 min), followed by 4% formaldehyde fixative (from paraformaldehyde heated to 60–65 °C) in a borate buffer (pH 9.5) at 4 °C (800–1000 ml for approximately 25 min). Immediately after perfusion, the brains were removed, postfixed for 2 h in the same fixative (supplemented with 20% sucrose), and transferred to 20% sucrose in 0.02 M potassium phosphate-buffered saline (KPBS). A series of 30-μm coronal sections (1:5 series) were cut on a freezing microtome and stored at −20 °C in antifreeze solution. To map MCH and *Fos* immunoreactivity and possible colocalization between these two markers, we first performed immunohistochemistry to label *Fos*-ir cells. One free-floating series of sections from each animal was pretreated with 0.3% hydrogen peroxide (Sigma Chemical, St. Louis, MO, USA) for 15 min, followed by 2 × 10 min rinses in KPBS and incubation in primary antibody against rabbit anti-*Fos* (1:70,000; Millipore, catalog # ABE 457 anti-*c-Fos* rabbit antibody polyclonal; for anti-*Fos* protocol vide [17]) in 3% normal goat serum diluted in KPBS + 0.3% Triton X-100, for ~24 h at room temperature. Subsequently, the sections were rinsed in KPBS (2 × 10 min) and incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (IgG; Vector Laboratories, Burlingame, CA, USA) diluted 1:800 in KPBS with 0.3% Triton X-100, then for 1 h at room temperature using peroxidase avidin–biotin complex solutions (Vector) diluted 1:333 in KPBS. The sections were then rinsed and subjected to immunoperoxidase reactions using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.02%; Sigma) with 0.003% hydrogen peroxide and nickel ammonium sulfate (NAS) at 2.5% dilute in a sodium acetate buffer 0.2 M, pH 6.5. After ~10 min, the reaction was terminated with successive rinses in KPBS. Afterward, the sections were subjected to the second immunohistochemistry protocol to label MCH cells. The sections were incubated in anti-MCH (1:70,000; PBL #234, kindly provided by Dr. J. Vaughan and Dr. Paul E. Sawchenko, Peptide Biology Laboratory, The Salk Institute, La Jolla, CA, USA). The specificity of the antibody was tested in previous studies [3, 5]. The antibody was diluted in 3% normal goat serum in KPBS + 0.3% Triton X-100 for ~24 h at room temperature. Subsequently, the sections were rinsed in KPBS (2 × 10 min) and incubated for 1 h at room temperature in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:800 in KPBS with 0.3% Triton X-100, then for 1 h at room temperature using peroxidase avidin–biotin complex solutions (Vector) diluted 1:333 in KPBS. The sections were then rinsed and subjected to immunoperoxidase reaction using 0.02% DAB (Sigma) with 0.003% hydrogen peroxide (but without NAS) diluted in a 0.2 M sodium acetate buffer (pH 6.5). After ~5 min, the reaction was terminated with successive rinses in KPBS. The sections were mounted on gelatin-coated slides, dehydrated, delipidated, and coverslipped with a DPX mounting medium. An intervening series was subjected to thionin staining for reference using the Nissl method to delineate the cytoarchitecture of the surrounding regions.

2.5. Stereology

Stereology was conducted by using a modified optical fractionator probe and stereological image analysis software (Stereo Investigator, MBF Bioscience, USA) operating a computer-driven stage motorized in the *x*, *y* and *z* axes (Ludl Electronic Products; Hawthorne, NY, USA) of an Eclipse 80i microscope (Nikon Instruments, Tokyo, Japan) equipped for brightfield and darkfield imaging. Using a low power magnifying lens, the areas to be counted were traced and the counting frames distributed in an unbiased, systematic and random way by the analysis software. Stained cells were counted using a 40× objective (0.95 NA).

To ensure homogeneity among different animals, the areas to be counted were drawn always following the same rules: the LHA is defined as the quadrangle formed by a superior horizontal line that passes at the top of the third ventricle (3V), an inferior horizontal line that crosses the fornix, a medial vertical line that also crosses at the fornix and a lateral line that follows the medial border of the internal capsule.

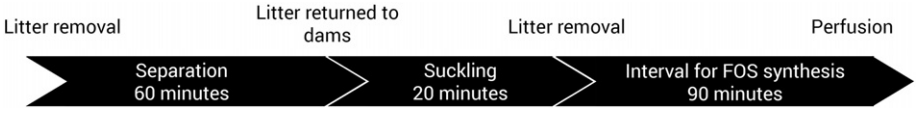

Fig. 1. Experimental design.