Testosterone Regulates Pro-Opiomelanocortin Gene Expression in the Primate Brain*

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ABSTRACT. Endogenous opioid peptides such as β-endorphin, derived from pro-opiomelanocortin (POMC), have been widely implicated as serving an important role in the neuroendocrine regulation of the primate reproductive axis. In both human and nonhuman primates, POMC neurons are thought to mediate, at least in part, the negative feedback action of sex steroids on GnRH. Sex steroids, such as testosterone, are thought to inhibit GnRH secretion by enhancing the inhibitory activity of β-endorphin; however, the cellular mechanisms by which steroid hormones regulate the activity of POMC neurons in the primate brain are unknown. In this study, we tested the hypothesis that testosterone stimulates POMC gene expression within the primate brain and that this regulation occurs within a specific subset of POMC neurons residing in the arcuate nucleus of the hypothalamus. We used in situ hybridization to compare cellular levels of POMC messenger RNA in intact (n = 4), castrated (n = 4), and castrated/testosterone-treated (n = 4) monkeys. We report that after castration of the male macaque (Macaca fascicularis), cellular POMC messenger RNA levels decline significantly (P < 0.05) in neurons within the arcuate nucleus and that this decline is prevented by replacement with physiological doses of testosterone. Moreover, we found that this testosterone-dependent modulation of POMC gene expression is restricted to a small fraction of the numerous POMC neurons located within the most anterior region of the arcuate nucleus in the brain of this primate species. These observations provide evidence that sex steroids regulate expression of the POMC gene in the primate brain. (Endocrinology 128: 1881–1886, 1991)

ENDOGENOUS opioid peptides in the brain are important neurotransmitters implicated in the regulation of numerous physiological functions, including feeding behavior, learning, analgesia, and immune responses (1). This, coupled with the observation made more than 50 years ago that female morphine addicts are often infertile or fail to have regular menstrual cycles (see Ref. 2), has generated considerable interest in understanding how endogenous opioids may be involved in the physiological regulation of reproductive function in humans. Moreover, the possibility that abnormalities in opioid peptide function may underlie the development of disorders of reproduction, such as chronic exercise- and stress-induced amenorrhea, has intensified studies of the regulation of opioidergic activity in the brain.

Received October 29, 1990.

The mammalian reproductive axis is coordinated by the hypothalamic secretion and trophic effects of GnRH, which itself is controlled by the negative feedback action of gonadal steroids operating through a mechanism that involves the opioid peptides (3–8). In the presence of gonadal steroids, β-endorphin acts through μ-receptors to inhibit GnRH secretion; however, this does not occur in an animal whose gonads have been removed. Although it is clear that gonadal steroids, such as testosterone, are required for the interaction between β-endorphin and GnRH neurons, the mechanisms by which testosterone influences the activity of β-endorphin neurons are largely unknown (9, 10). The effects of endogenous opioid peptides on reproduction have been described in a number of mammalian species; however, the neuroendocrine reproductive axis of the primate is unique in several important respects, including the anatomical organization of the hypothalamic neurons involved in the neuroendocrine regulation of GnRH secretion (11–13). The primary objective of this study was to test, in the primate brain, whether testosterone stimulates expression of the POMC gene, which encodes the precursor of β-endorphin. A secondary objective was to determine whether an effect of testosterone might be restricted to a subpop-
ulation of POMC neurons from among the large number of POMC neurons within the arcuate nucleus of the brain.

Materials and Methods

Animal care and surgical procedures

Adult male macaques, Macaca fascicularis, were used for these studies. Animals were cared for at the Regional Primate Research Center at the University of Washington in accordance with institutional guidelines. They were housed in individual cages under controlled conditions of heat (25.5 ± 1°C), humidity (65%), and light (on 0600 h; off 1800 h). Animals were fed Purina monkey chow and given water ad libitum and received fresh fruit twice, and chewable vitamin and iron tablets once weekly. Adult animals weighed 4.5-9.0 kg and were sexually mature as assessed by plasma testosterone levels.

Castorizations were performed under aseptic conditions on animals anesthetized with fluothane. At the time of castorization, animals received either blank Silastic (0.465 cm, Dow-Corning Co., Midland, MI) capsules or capsules containing powdered testosterone (Sigma Chemical Co., St. Louis, MO) packed to a final length of 4 cm, and implanted in a sc pocket in the midscapular region, where they remained for 3 weeks, until the time of death. Blood samples were taken by saphenous venipuncture at the time of castorization and again at the time of death, and used for the evaluation of testosterone levels.

Tissue preparation

Before the perfusion was complete, the head was removed and placed in a Kopf (Tujunga, CA) stereotactic instrument. Using a stainless steel cannula mounted on the stereotaxic instrument, we made bilateral tracks in the brain at 28 mm and 30 mm anterior to ear-bar-zero, 7 mm off midline on each side. This provided a means of aligning the hypophalamic block during subsequent sectioning so that sections in a frontal plane could be obtained. The hypothalamus and basal forebrain was blocked by making cuts at the inflection point of the olfactory nerves (anterior), corpus callosum (superior), posterior to the mammillary bodies (posterior), and 9 mm from the midline bilaterally. The block was frozen on dry ice and then stored at −80°C until slicing. Frontal sections (30 μm), cut at −18°C were mounted onto cold poly-L-lysine (50 μg/ml; Sigma, St. Louis, MO)-coated glass slides. For analyses of POMC messenger RNA (mRNA) containing cells, tissue sections (200 μm apart) throughout the arcuate nucleus were processed for in situ hybridization.

POMC complementary RNA probe synthesis

A 993-basepair ribonucleotide probe, derived from a Macaca nemestrina pituitary complementary DNA (cDNA) library, was transcribed in the presence of 8S-α-thio-uridine triphosphate (New England Nuclear Bosten, MA) to a final specific activity of 1–2 × 10⁷ dpm/μg RNA. After purification, the RNA was hydrolyzed in 100 mM bicarbonate buffer (pH 10.2) to yield fragments approximately 150 bases in length. On the day of the assay, the riboprobe (final concentration, 1.2 μg/ml-kilobase) was mixed in hybridization buffer (final concentrations in this mixture were: 500 μg/ml total yeast RNA, 50% deionized formamide, 0.3 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM diethytothreitol (DTT), 10% dextran sulfate, 1X Denhardt’s).

In situ hybridization

The tissue received the following treatment immediately before application of the probe: postfixation (3 min) in 4% paraformaldehyde in 0.1 M PB, two 5-min washes in PB, proteinase K (0.5 mg/ml PK in 0.1 M Tris-HCl pH 8.0, 50 mM EDTA, 30 min at 37°C), acetic anhydride (0.25% in 0.1 M triethanolamine pH 8.0 for 10 min at 25°C), 2× SSC (3 min at 25°C; 1× SSC = 150 mM NaCl, 15 mM sodium citrate), dehydration through graded ethanol, delipation in chloroform (5 min), and rehydration through 95% ethanol. Tissue was air dried from ethanol, and 45 μl/hypothalamic slice of the probe mixture was applied to dry tissue. Parafilm was used as a coverslip, and the edges of the parafilm were sealed with rubber cement. Slides were incubated in a moist chamber overnight at 60°C. The next day, parafilm coverslips were pulled off with forceps, and the slides were rinsed briefly in 4× SSC/10 mM DTT. The slides were then rinsed in fresh 4× SSC/10 mM DTT for 15 min at ambient temperature, treated with RNase A (30 μg/ml in 10 mM Tris pH 8.0, 1 mM EDTA, 0.5 M NaCl for 39 min at 37°C), stirred 1 h in 2× SSC/2 mM DTT/20% deionized formamide at 60°C for 30 min. The slides were then dehydrated through graded ethanol (50%, 80%, and 100%; the first two alcohols contained 300 mM ammonium acetate), then air dried. The slides were dipped in photographic emulsion (Kodak NTB-2, Eastman Kodak, Rochester, NY, diluted 1:1 with 500 mM ammonium acetate), air dried for 30 min, placed in a moist chamber for 3 h, and dried again on the bench for 45 min. Slides were exposed in light-tight boxes at 4°C for 6.5 days, developed (Kodak D-19, diluted 1:1 with distilled water) for 4 min and fixed (Kodak fixer) for 10 min at 15°C, rinsed 30 min in running tap water, and counterstained with cresyl violet.

Image analysis

Tissue sections 200 μm apart throughout the rostral-caudal extent of the arcuate nucleus were processed for in situ hybridization. Tissue was visualized under darkfield illumination to identify clusters of photographic grains overlying cells, and lightfield illumination was used to verify that a cresyl-stained
nucleus was associated with each cluster. Twenty pro-opiomelanocortin (POMC) mRNA-containing cells were analyzed on each tissue slice. Sixteen slices from each animal were analyzed, so that a total of 320 cells per animal were subjected to an automated grain detection and quantification process by use of a computerized image processing system (14). Before analysis, the slides were anatomically matched among animals, then were randomized and assigned a 3-letter code so that the operator was blinded to the slide’s treatment group assignment. The total number of photographic grains overlying a cell, corrected for background, is referred to as the number of grains per cell. An arithmetic mean number of grains per cell was calculated for each animal, and this mean was used in a one-way analysis of variance to test for differences between treatment groups. All variance measures are ±SEM, calculated with \( n = 4 \), representing the number of animals in each treatment group.

Probe specificity

Incubation of tissue sections with a mixture of unlabeled and labeled riboprobe in a ratio of 100:1 (unlabeled:labeled) was performed to control for nonspecific binding; grain clusters were not apparent on these sections.

Testosterone assay

Testosterone levels in ether-extracted serum samples were measured by RIA with an antiserum raised against testosterone linked at the 3-position by carboxymethoxime to BSA. The antiserum exhibited a cross-reactivity of 14% with dihydrotestosterone, 6% with \( \alpha \)-androstenediol, and 2% or less with all other steroids tested. The minimum sensitivity of the assay was less than 0.1 ng/ml, and the intra- and interassay coefficients of variation were 3.0% and 8.5%, respectively.

Results

We compared the relative cellular levels of POMC mRNA signal, expressed as grains per cell, in the brains of male crab-eating macaques (Macaca fascicularis) that were either intact (\( n = 4 \)), castrated (\( n = 4 \)), or castrated and implanted with Silastic capsules designed to maintain physiological levels of testosterone in the peripheral circulation (castrate + T) (\( n = 4 \)). The majority of POMC neurons in the primate brain were observed to be located in two large clusters, residing on both sides of the tip of the third ventricle within the arcuate nucleus (Fig. 1). The overall POMC mRNA signal levels in the arcuate nucleus were: intact, 152.3 ± 4.9 grains per cell; castrate, 111.7 ± 11.9 grains per cell; castrate + T, 157.3 ± 14.0 grains per cell. Thus, when POMC cells throughout the arcuate nucleus were analyzed, we observed that castration resulted in a 27% decrease in the mean number of grains per cell over POMC mRNA-containing cells compared with cells from intact controls (\( P < 0.05 \)). We also observed that testosterone replacement prevented this decrease in POMC message, as there was no statistical difference in grains per cell between intact and castrate + T animals (Figs. 2 and 3).

To determine whether the effects of castration and testosterone treatment occur only in particular subsets of POMC neurons, we divided the arcuate nucleus into four divisions of equal length (about 0.8 mm each) along the anterior-posterior axis. The anterior-posterior coordinates of these regions correspond roughly to those of the plates shown in Fig. 1, defining the anterior boundary of the nucleus as the point at which the optic chiasm first indents the midline, and the posterior boundary as the point of appearance of the fourth ventricle. The castration-induced decrease in cellular POMC mRNA signal levels was most pronounced in the most rostral
Fig. 3. The effect of castration and steroid replacement on the mean cellular POMC mRNA levels throughout the entire arcuate nucleus of intact (n = 4), castrated (n = 4), and castrated + T (n = 4) adult male macaques.

Fig. 4. The effect of castration and testosterone replacement on cellular POMC mRNA levels in four regions of the arcuate nucleus of male macaques (P < 0.05). As described in the text, the nucleus is divided into four divisions of equal length along the anterior-posterior axis and designated ArcA, ArcB, ArcC, and ArcD such that ArcA is the most anterior and ArcD is the most posterior portion of the nucleus.

180.0 ± 29.8 grains per cell. Changes in POMC mRNA signal levels associated with castration were not statistically significant in the other three divisions of the arcuate nucleus (Fig. 4).

The effectiveness of the castration and testosterone replacement regimens was demonstrated by the mean serum testosterone levels in each group, which were: intact, 4.8 ± 0.8 ng/ml; castrated, 0.25 ± 0.07 ng/ml; and castrated + T, 8.6 ± 0.4 ng/ml. The testosterone values in castrated + T animals is within the range of normal testosterone values for intact adult males (15).

**Discussion**

We have shown that expression of the POMC gene in the hypothalamic arcuate nucleus is reduced after castration can be the hypotension effect mode low, the small applic has n (16–2) such action have a similar circuit. The per se not b hypotractions to an acute hypot. latter teract to the POMC one of Testor to infl express comp preser. In the neuron has no effect from the testostoner arcuate rat (24 studies to the haps t POMC.
tation of adult male macaques, and that this decrease can be prevented by administration of physiological levels of testosterone. Our data are consistent with the hypothesis that the β-endorphin system requires the presence of gonadal steroids to exert its physiological effects on the reproductive system. According to this model, when circulating levels of gonadal steroids are low (e.g., after castration or during the pubertal state), the inhibition of GnRH neurons by β-endorphin is so small that its functional removal (by the pharmacological application of a μ receptor antagonist, such as naloxone) has no perceptible effect on GnRH secretory activity (16–20). The present study provides an explanation for such an attenuation of β-endorphin activity: the castration-induced reduction in POMC gene expression we have observed may account for a diminution of β-endorphin secretory activity that persists in states of low circulating sex steroids.

The effects of testosterone on β-endorphin secretion per se are not well characterized in any species, and have not been described at all in the primate brain. The hypothalamic content of β-endorphin increases after castration of male rats, but this could be attributed either to an increased synthesis or decreased release of the peptide; an in vitro reduction in β-endorphin release from hypothalamic slices of castrated male rats favors the latter explanation (21, 22). Because POMC neurons interact with a variety of physiological systems in addition to the reproductive axis, it cannot be asserted that the POMC neurons we analyzed make functional connections with GnRH neurons. Nevertheless, the data presented here are consistent with the proposed model of POMC interaction with the reproductive system.

The manner in which testosterone exerts its effect on POMC gene expression is also unknown, since testosterone can act on the genome through multiple pathways. Testosterone itself may act through androgen receptors to influence a variety of cellular events, including gene expression, or it can be metabolized to other steroid compounds (for example, to dihydrotestosterone in the presence of 5α-reductase or to estradiol by the action of aromatase) which themselves exert physiological effects. In the rat, it appears that testosterone acts on POMC neurons through conversion to estradiol (23), but this has not yet been established in a primate species.

This study in the primate brain, as well other studies from our lab in the rat brain documenting the effects of testosterone on POMC mRNA levels in neurons of the arcuate nucleus, are at variance with other reports in the rat (24, 25). Several important differences between these studies and the present study bear discussion, in addition to the obvious consideration of species differences. Perhaps the most salient point is that we measured mean POMC mRNA levels in individual neurons, whereas in the other studies, changes in POMC mRNA relative to total mRNA were measured by solution hybridization. It is conceivable that after castration the POMC mRNA content of individual neurons in the anterior part of the arcuate decreases, but the total number of neurons expressing POMC mRNA (and thus the amount of hypothalamic POMC mRNA) in the arcuate increases. Additionally, there could be a decrease in the amount of total mRNA in the hypothalamus, or some other change in baseline levels of mRNA which could decrease the standard to which POMC mRNA levels are compared. These discrepancies are not yet resolved, but one important consideration may be the heterogeneity of POMC neurons in the hypothalamus.

Our demonstration that castration induces a greater decrease in POMC mRNA in the anterior part of the arcuate nucleus emphasizes this heterogeneity. Regional specificity of steroid effects on POMC gene expression has also been demonstrated in the rat (26, 27) and could result from regional variations in steroid hormone receptors, steroid metabolism, or in steroid-sensitive afferent inputs to these neurons. Indeed, it has been shown in the female rat hypothalamus that of those neurons which are immunoreactive for β-endorphin, only about 30% have progesterone receptors (28), and something less than 30% have estrogen receptors (29, 30). Thus, there are distinct subpopulations of POMC neurons upon which steroid hormones could act directly. There is also heterogeneity in the projections of POMC neurons from different regions of the arcuate. Although careful mapping studies have not been performed in the macaque brain to indicate where POMC neurons within the arcuate study, studies in the rat have examined the projections of cells containing ACTH, another cleavage product of the POMC precursor molecule. They show that a population of ACTH-containing cells whose soma are in the arcuate nucleus project to the medial preoptic area, and that in the medial preoptic area there are numerous synaptic connections between ACTH neurons and GnRH neurons (31, 32). Functional heterogeneity is not unique to the POMC neurons within the arcuate but is evident in other systems as well, including the dopaminergic cells in the arcuate (33, 34). These observations underscore the fact that the functional organization of the brain defies dissections along classical anatomical boundaries and suggests that multiple criteria must be used to define and study the regulation of neurotransmitter systems, even within individual brain nuclei.

Acknowledgments

We thank Pam Kolb, Emilia Kabigtin, and the staff of the Regional Primate Research Center at the University of Washington for excellent technical support. We also thank Dr. Stanley Watson for providing us...
with the Macaca nemestrina POMC cDNA, and Dr. William Brenner for supervising the testosterone assays.

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