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Potential Role of Maternal Progesterone in the Sexual Differentiation of the Brain

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ABSTRACT
In rats, fetal testosterone directs sexual differentiation of the brain. However, fetuses are also exposed to maternal progesterone. Here we report that progesterin receptor immunoreactivity in the medial preoptic nucleus (MPN) of fetal and neonatal rats is high in males but virtually absent in females. The MPN is one of the most sexually dimorphic structures in the rat brain and mediates several sexuallly differentiated behaviors. This suggests that progesterone may play a previously overlooked role in the development of sex differences in the brain and behavior. Henceforth, a novel function of the mother in the sexual differentiation of the CNS must be considered.

Sex differences in fetal and neonatal testosterone levels determine the sexual differentiation of the brain (1,2). Fetuses are also exposed to maternal hormones that readily cross the placenta and are presumably present in mother’s milk. The levels of maternal progesterone undergo rapid and extreme changes at specific times during pregnancy and lactation. Progesterone levels during pregnancy reach peak levels at least three times higher than those of non-pregnant females and decline dramatically just prior to parturition (3,4). Progesterone in fetuses and pups appears to be of maternal origin because maternal and fetal blood progesterone levels are correlated on the last four days of pregnancy and do not differ between fetal males and females (5,6). Moreover, the developing ovary does not produce steroids until late postnatal life (7,8,9). It is not precisely known whether the developing brain is sensitive to progesterone. Progesterone binding can be detected in homogenized hypothalamus two days after birth, but not earlier (10). PR mRNA can be detected at least two days prior to birth (11) but it is not known whether this message is translated into functional protein, nor is it known whether there is differential sensitivity of specific brain structures to progesterone between the sexes. To answer these questions, we examined the expression of PR immunoreactivity (PR-ir) in the brains of perinatal male and female rats.

Materials and Methods
Tissue preparation
Female Sprague-Dawley rats (60-80 days of age, Taconic, Germantown, NY) were mated and the day copulatory plugs were found was considered day 1 of gestation (E1). Females were allowed to deliver their pups normally on E23. Two pups of each sex were randomly selected from each of three litters on the day of birth. When a sex difference in PR-ir was discovered postnatally, a second experiment was performed to determine when this sex difference develops. Therefore, fetuses were removed from additional pregnant females by caesarean section on E18, E20 and E21. Pregnant females were overanesthetized with pentobarbital:chloral hydrate, the abdominal cavity was opened, and the uterine horns were removed and placed on ice. Fetuses were removed from the uterine horns and undercooled with ice until decapitation. Because the sex of E18 animals could not be determined visually, a total of 8 randomly selected animals were run. Pups and fetuses were anesthetized by undercooling them with ice for approximately 5 minutes and then killed by decapitation. The brains were removed, placed in fixative and prepared for immunocytochemistry. Brains were placed in 5% acrolein in 0.1M phosphate buffer, pH 7.6, for approximately 12 hours, rinsed overnight in 0.05M tris/HCl in 0.9% NaCl (TBS; pH 7.6) then cryoprotected in 30% sucrose in TBS for 48 hours. Transverse sections were cut at 50um on a freezing microtome and stored in cryoprotectant at -20°C.

Immunocytochemistry
Immunocytochemistry was performed using PR antiserum DAKO (Glostrup, Denmark), which is a rabbit polyclonal antibody directed against the DNA binding domain of the human progesterone receptor and detects both the A and B forms of the receptor (12). All incubations were performed at room temperature unless otherwise indicated. Sections were rinsed in TBS three
times for 5 minutes to remove all cryoprotectant solution. Sections were then incubated in 1% sodium borohydride in TBS for 10 minutes. The sections were rinsed in TBS six times for 5 minutes each and then incubated in TBS containing 20% normal goat serum (NGS), 1% H₂O₂ and 1% bovine serum albumin for 30 minutes. Progesterone receptor antiserum was diluted (1:1000 for neonatal tissue; 1:500-1:100 for fetal tissue) in TBS (pH 7.6 at 4°C) containing 1% NGS, 0.5% Triton X-100, 0.1% gelatin and 0.02% sodium azide for 40 hours at 4°C. Following 3 rinses (5 minutes each) in TBS containing 0.02% TritonX-100, 0.1% gelatin and 0.02% sodium azide, the sections were incubated for 90 minutes in biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Burlingame, CA) at a concentration of 5μg/ml in TBS containing 1.5% NGS, 0.02% Triton X-100, 0.1% gelatin and 0.02% sodium azide. After 2 rinses (5 minutes each) in TBS containing 0.02% Triton X-100, 0.1% gelatin, 0.02% sodium azide and 2 rinses (5 minutes each) in TBS, the sections were incubated for 90 minutes in the ABC reagent Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). After 3 rinses (5 minutes each) in TBS, the sections were incubated in TBS containing 0.05% dianisidobenzidine, 0.75mM nickel ammonium sulfate, 0.15% B-D-glucose, 0.04%ammonium chloride and 0.001% glucose oxidase for approximately 20 minutes. The sections were then rinsed 3 times (5 minutes each) in TBS and then mounted on gelatin-coated slides and coverslipped with Permount (Fisher Scientific). Preadsorption of the PR antiserum overnight with a 10-fold excess of human progestrone receptor (A and B forms) or omission of the PR antiserum abolished all immunoreactivity.

**Analysis**

Sections through the rostral MPN (as in coronal plate 8:220 of Altman and Bayer (13)) were chosen from each animal. The relative amount of PR-ir was determined for each section through the MPN using a computer-assisted image analysis system and the NIH IMAGE program. The relative amount of PR-ir was defined as the area (μm²) within the MPN covered with pixels using gray level thresholding. Comparisons between the sexes in postnatal animals were made using the Student’s t test (p<0.05) and in prenatal animals using analysis of variance (ANOVA; p<0.05). Post-hoc analyses were performed using the Student-Neuman Keuls test (p<0.05).

**Results**

PR-immunoreactive cells were easily detected in the brains of both males and females on the day of birth and on prenatal days E20 and E21. Brain regions containing PR-immunoreactive cells included the MPN, the bed nucleus of the stria terminals, the posterior paraventricular nucleus, the ventromedial nucleus of the hypothalamus, the lateral hypothalamus, the basolateral nucleus of the amygdala, the dentate gyrus of the hippocampus, and neocortex.

After finding significantly more PR-immunoreactive cells in the MPN of males than females on the day of birth (p<0.001) (Figures 1 and 2A), we examined when this sex difference emerges prenatally. As on the day of birth, there was a significant sex difference in PR-ir prenatally as well (ANOVA, p<0.0003, F=44.2, df=1).

![Figure 1](https://example.com/figure1.png)

Figure 1. Progesterone receptor immunoreactivity in the medial preoptic nucleus of male and female neonatal rats on the day of birth.
During development than the female brain. The adult MPN has numerous sex differences, e.g. in cell density, neurotransmitter content and synaptic density, that are the result of differential exposure to testicular testosterone and its metabolite estradiol during the perinatal period (1,2). The MPN is also critically involved in several behaviors which are sexually differentiated in the rat, such as sexual behavior and maternal behavior (14,15). The current study suggests that the progestin receptor may play an important role in the development of these neural and behavioral sex differences.

As PR-ir is first expressed in the male MPN after E18, while in many other regions PR is present on E18 and possibly earlier, levels of PR-ir in the MPN may be uniquely regulated by a factor not controlling PR-ir expression in other regions at this age. A good candidate is the testosterone metabolite, estradiol, which induces PR expression in the brains of adult males and females (16). If testosterone regulates PR expression in the fetal MPN, the presence of a testosterone surge in males (5,6) and the absence thereof in females would explain a sex difference in PR expression. Indeed, preliminary data suggests that the sex difference in PR-ir in MPN can be abolished by treating females with testosterone propionate prenatally. The onset of the testosterone surge at E18 (5,6) correlates with the presence of a sex difference in PR expression on E20, which is possibly the earliest detectable sex difference in a specific region of the rat brain.

Progestins given during pregnancy have been linked to altered sexual differentiation of behavior in rats (17,18,19). However, the findings of the present study may have clinical implications as well. The children of women treated with progestins during pregnancy for the prevention of miscarriage exhibit a variety of physical, psychological and behavioral differences compared to unexposed subjects (20,21,22,23). Previously, the effects of progestins on development have been attributed to the stimulation of androgen receptors by high doses of progesterone and synthetic progestins given to these women, some of which have androgen receptor agonist activity. However, the results of the present study suggest an alternative interpretation of these clinical findings. Progestins may act directly at progesterone receptors, thereby influencing the effects of testosterone on the sexual differentiation of the brain.

Discussion

The higher level of PR-ir in the MPN of males suggests that male brains are substantially more susceptible to the effects of maternal progesterone

![Figure 2. The amount of PR-ir (area covered by pixels) within the medial preoptic nucleus of male and female rats on (A) E20 and E21 or (B) the day of birth. * significantly different from males of the same age.](image-url)
Acknowledgments

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