Sex Differences in Progesterone Receptor Immunoreactivity in Neonatal Mouse Brain Depend on Estrogen Receptor α Expression

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ABSTRACT: **Around the time of birth, male rats express higher levels of progesterone receptors in the medial preoptic nucleus (MPN) than female rats, suggesting that the MPN may be differentially sensitive to maternal hormones in developing males and females. Preliminary evidence suggests that this sex difference depends on the activation of estrogen receptors around birth. To test whether estrogen receptor alpha (ERα) is involved, we compared progesterone receptor immunoreactivity (PRir) in the brains of male and female neonatal mice that lacked a functional ERα gene or were wild type for the disrupted gene. We demonstrate that males express much higher levels of PRir in the MPN and the ventromedial nucleus of the neonatal mouse brain than females, and that PRir expression is dependent on the expression of ERα in these regions. In contrast, PRir levels in neocortex are not altered by ERα gene disruption. The results of this study suggest that the induction of PR via ERα may render specific regions of the developing male brain more sensitive to progesterone than the developing female brain, and may thereby underlie sexual differentiation of these regions.** J Neurobiol 47: 176–182, 2001

Keywords: **knock-out; medial preoptic nucleus; ventromedial nucleus; development**

INTRODUCTION

The majority of morphological sex differences in the mammalian nervous system depend on differential perinatal exposure of developing males and females to the gonadal steroid hormone testosterone. In many instances, it is the action of estradiol, converted within the brain from testosterone by the enzyme aromatase, that is responsible for the masculinization of brain development (Gorski, 1985). Estrogen receptors (ER) are an important component in sexual differentiation because ER blockers are capable of diminishing the masculinization of both the brain and behavior (McEwen et al., 1977). However, the lack of specificity of these receptor blockers has impeded the determination of the relative roles of the classical estrogen receptor alpha (ERα) and a more recently characterized ERβ (Kuiper et al., 1996, 1997; Tremblay et al., 1997) in the sexual differentiation of the brain.

The development of ERα "knockout" (ERαKO) mice that are homozygous for an insertional disruption of the ERα gene (Lubahn et al., 1993; Couse et al., 1995) allows an alternative method to address this question. These mice have drastically reduced levels of ERα immunoreactivity in the hypothalamus

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(Ogawa et al., 1997; Rissman, 1997). They do, however, express ERβ mRNA in the hypothalamus (Shughrue et al., 1997b), which is translated into a protein that binds estrogen (Shughrue et al., 1999). Very few studies thus far have examined the importance of ERα in the sexual differentiation of the brain. Simerly et al. (1997) demonstrated that dopamine cell number in the anteroventral periventricular nucleus of the hypothalamus is feminized in ERαKO adult male mice. In addition, McCarthy et al. (1993) demonstrated a clear role for ERα in the masculine development of the sexually dimorphic nucleus of the preoptic area using antisense DNA to inhibit specifically the translation of ERα, but not ERβ, mRNA. These studies clearly reveal an important role for ERα in the sexual differentiation of these areas. Thus far, the mechanisms of action for ERα during the developmental critical period for sexual differentiation have not been elucidated.

The sexually dimorphic expression of progesterone receptor immunoreactivity (PRir) in the developing medial preoptic nucleus (MPN; Wagner et al., 1998a) may lend itself well for such an analysis. In developing male rats, levels of PRir in the MPN are high between embryonic day 19 and postnatal day 8, whereas in female rats PRir is virtually absent in the MPN during this time. In the brain of adult rats and mice, the expression of progesterone receptors (PR) in the MPN is induced by estradiol (MacLusky and McEwen, 1978; Shughrue et al., 1997a; Moffatt et al., 1998), and results from our laboratory suggest that the estrogenic metabolite of testosterone, estradiol, but not the androgenic metabolite, dihydrotestosterone, induces PR expression in the MPN of fetal and neonatal male rats (Wagner et al., 1998b).

Both ERα and ERβ could contribute to PR expression in the neonatal rat brain. Both receptors are expressed in the rodent preoptic area during development (DonCarlos, 1996, 1997; Yokosuka et al., 1997; Karolczak and Beyer, 1998), and given that estradiol treatment increases PRir in adult ERαKO mice, ERβ may play at least a small role in the expression of PR in the adult brain (Shughrue et al., 1997a; Moffatt et al., 1998). Moreover, opposing sex differences in ERα and ERβ expression in the preoptic area during development (DonCarlos and Handa, 1994; DonCarlos, 1996; Karolczak and Beyer, 1998) suggest differential roles of these two receptor types in the sexual differentiation of the MPN. Because preliminary data from our laboratory confirmed that mice, like rats, show a sex difference in PRir in the MPN, the present study examined the importance of ERα for the sex difference in expression of PR in the MPN by comparing neonatal ERαKO and wild-type mice. This experiment examined two additional brain regions, the caudal ventromedial nucleus of the hypothalamus (cVMN), in which PR expression depends on estradiol in adults (MacLusky and McEwen, 1978; Shughrue et al., 1997a; Moffatt et al., 1998), and the neocortex, in which PR expression appears to be independent of estradiol in the neonatal mouse (Shughrue et al., 1991a). The purpose of this study was to elucidate the role of ERα in the generation of a neural sex difference that exists transiently during development.

MATERIALS AND METHODS

Tissue Preparation

Female C57BL/6J mice heterozygous for a targeted disruption of the ERα gene were mated with heterozygous male counterparts. Animals were housed in the Wyeth-Ayerst animal care facility (AAALAC certified) with a 12-h light/dark photoperiod and free access to water and rodent chow. All animal procedures used in this experiment were approved by the Institutional Animal Care and Use Committee at Wyeth-Ayerst. Following mating, females were allowed to gestate and deliver pups normally. Pups generated from these females were sacrificed by decapitation on either the day of birth (postnatal day 0; P0), P4, or P8. Brains from P4 and P8 pups were removed from the skull, while P0 brains were left in the skull and immersion fixed in 5% acrolein in 0.1 *M* phosphate buffer (pH 7.6) overnight. Brains were rinsed overnight in 0.1 *M* phosphate-buffered saline (PBS) and then cryoprotected in 30% sucrose in PBS. Pups were genotyped using PCR analysis of DNA from tail samples as previously described (Lubahn et al., 1993). Brains from male and female pups that were either wild type (WT, +/+) or possessed two copies of the disrupted ERα gene (KO, −/−) were used in this study. The number of animals used for each group was as follows: male WT, P0 = 5, P4 = 4, P8 = 4; male KO, P0 = 4, P4 = 5, P8 = 4; female WT, P0 = 5, P4 = 5, P8 = 5; female KO, P0 = 4, P4 = 5, P8 = 5. Brains were sectioned on a freezing microtome at 50 μm in the coronal plane and stored in cryoprotectant [30% sucrose, 1% polyvinyl pyrrolidone, 30% ethylene glycol in 0.1 *M* phosphate buffer (pH 7.6)] at −20°C.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Wagner et al., 1998a) using a rabbit polyclonal antiserum (DAKO; Glostrup, Denmark) that is directed against the DNA binding domain of the human progesterone receptor and recognizes both the A and B forms of the receptor, and binding of the antisera to PR is not influenced by ligand binding (Traish and Wotiz, 1990; unpublished observation). In short, sections were rinsed, incubated in 1% sodium borohydride in 0.05M Tris-buffered saline (TBS;

pH 7.6) for 10 min and incubated in TBS containing 20% normal goat serum (NGS), 1% H_2O_2, and 1% bovine serum albumin for 20 min. Sections were incubated for 72 h at 4°C in progesterone receptor antiserum diluted (1:500) in TBS containing 1% NGS, 0.5% Triton X-100, and 0.02% sodium azide. Following three rinses in TBS, the sections were incubated for 90 min in biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Burlingame, CA) at a concentration of 5 μg/mL in TBS containing 1.5% NGS, 0.03% Triton X-100, and 0.02% sodium azide. After several rinses in TBS, the sections were incubated for 90 min in the ABC reagent (Vectastain Elite Kit, Vector Laboratories). The sections were then incubated in TBS containing 0.05% diaminobenzidine, 0.75 m*M* nickel ammonium sulfate, 0.15% β-D-glucose, 0.04% ammonium chloride, and 0.001% glucose oxidase for approximately 20 min, then rinsed in TBS and mounted on gelatin-coated slides. Preabsorption of the PR antiserum overnight with A and B forms of the human progesterone receptor at a molar concentration 10 times higher than the primary antibody concentration, or omission of the PR antiserum abolished all immunoreactivity.

Analysis

One section from each animal through the rostral MPN (Coronal Figure 9; Altman and Bayer, 1995) and one section through the cVMN (Coronal Figure 15; Altman and Bayer, 1995) were anatomically matched across animals and were chosen for analysis by an experimenter blind to treatment group. All PRir in neocortex in a single section was analyzed in the same section as that selected for the rostral MPN. In the mouse, PRir is largely found in Layer V of primary somatosensory cortex. Microscopic images of the PRir in the MPN, cVMN, and neocortex were captured with an Olympus BH-2 microscope fitted with a CCD72 (Dage MTI, Michigan City, MI) camera connected to a QuickCapture frame grabber board (Data Translation Inc., Marlboro, MA) in a MacIntosh IIfx computer. NIH Image software (W. Rasbaud, National Institutes of Health, Bethesda, MD) was used to analyze captured images. The total amount of PRir in each region (MPN, cVMN, cortex) was determined by measuring the area (μm^2) covered by "thresholded" pixels [i.e., those pixels with a gray level higher than a defined threshold density (specific immunoreactive staining)]. "Threshold" was defined as the mean density five times the standard deviation higher than the mean background density. The mean background density was measured in a region devoid of PRir immediately lateral to the analyzed region containing PRir. Statistical analysis was performed using a three-way analysis of variance [ANOVA; $p < 0.05$; factors = age (3), X sex (2), X genotype (2)], followed by planned pairwise comparisons using Fishers Least Significant Difference (LSD) *post hoc* analysis ($p < 0.05$).

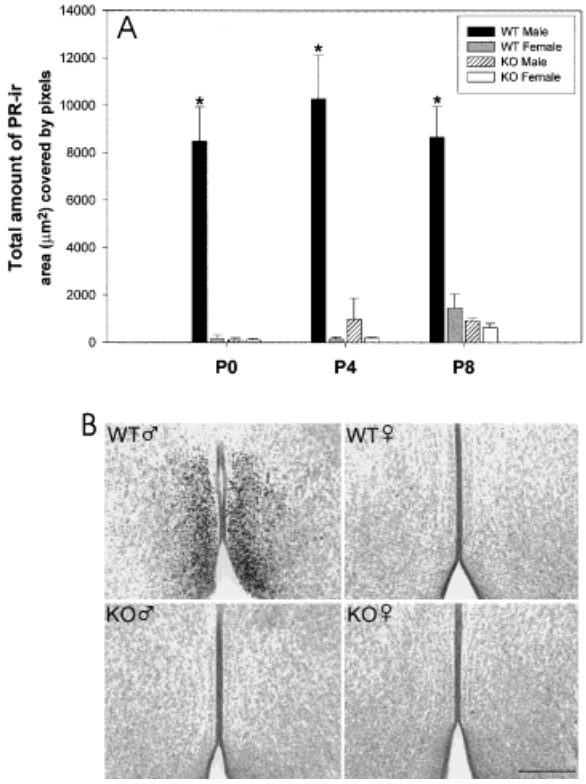

Figure 1 (A) The relative total amount of progesterone receptor immunoreactivity (PRir) in the medial preoptic nucleus (MPN) on postnatal day (P) 0, 4, and 8 in male and female mice that were either wild-type (WT) or were estrogen receptor α "knock outs" (KO). * significantly different from all other groups of the same age. (B) PRir in the MPN of P4 male and female mice that were either WT or KO. Bar = 200 μm.

RESULTS

Medial Preoptic Nucleus

Males had much higher levels of PRir in the MPN than females at all postnatal ages examined; however, this sex difference was only present in WT mice [Figs. 1 and 2(A)]. ANOVA revealed a significant main effect of sex [$p < 0.001$; $F_{(1,52)} = 104.6$] and genotype [$p < 0.001$; $F_{(1,52)} = 100.8$], and a significant interaction between sex and genotype [$p < 0.001$; $F_{(1,50)} = 88.3$]. There was no main effect of age. *Post hoc* analysis showed that WT males had significantly more PRir in MPN than WT females ($p < 0.001$ for P0, P4, and P8) or ERαKO males ($p < 0.001$ for P0, P4, and P8). ERαKO males and ERαKO females did not differ from each other at any age examined.

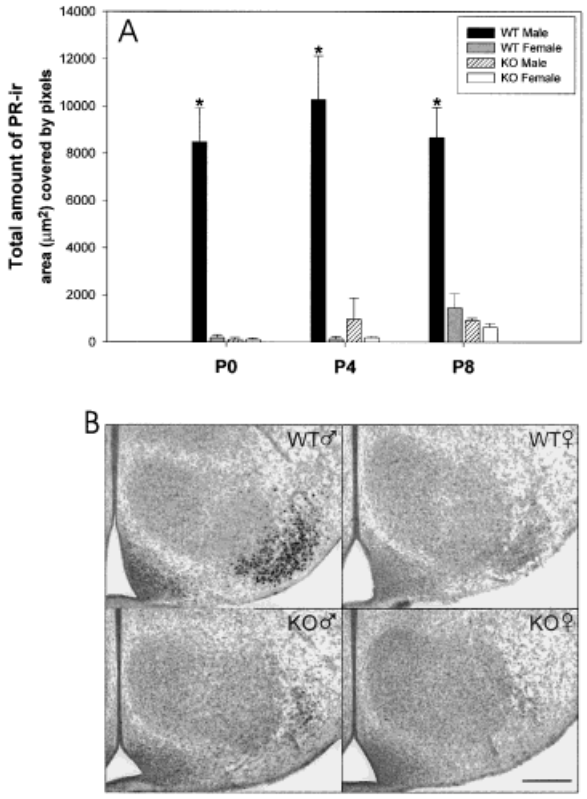

Figure 2 (A) The relative total amount of progesterone receptor immunoreactivity (PRir) in the ventromedial nucleus (VMN) on postnatal day (P) 0, 4, and 8 in male and female mice that were either wild-type (WT) or were estrogen receptor α "knock outs" (KO). * significantly different from all other groups of the same age. (B) PRir in the VMN of P4 male and female mice that were either WT or KO. Bar = 200 μm.

Ventromedial Nucleus

As in the MPN, males had much higher levels of PRir in the cVMN than females at all postnatal ages examined, and this sex difference was present only in WT mice [Fig. 2(B)]. There were significant main effects of sex [$p < 0.001$; $F_{(1,52)} = 69.4$], genotype [$p < 0.001$; $F_{(1,52)} = 45.4$], and age [$p < 0.05$; $F_{(2,51)} = 3.8$]. There were significant interactions between sex and genotype [$p < 0.001$; $F_{(1,50)} = 48.3$] and between sex and age [$p < 0.05$; $F_{(2,49)} = 3.3$]. There were no significant age/genotype or sex/age/genotype interactions. *Post hoc* analysis showed that WT males had significantly more PRir in cVMN than WT females ($p < 0.001$ for P0, P4, and P8) or ERαKO males ($p < 0.001$ for P0, P4, and P8). ERαKO males and ERαKO females did not differ from each other at any age examined. In addition, WT males had significantly higher levels of PRir in the cVMN on P4 than on P0 ($p < 0.01$) or P8 ($p < 0.005$). However, there were no significant age effects in WT or ERαKO females or in ERαKO males.

Cortex

The total amount of PRir in cortex did not vary by ERα genotype (Fig. 3). There were no significant main effects of sex, age, or genotype. There was a significant interaction between sex and age [$p < 0.05$; $F_{(1,32)} = 5.67$], but no other significant interactions. WT females had significantly more PRir in cortex than WT males on P8 ($p < 0.05$), but did not significantly differ from males on P4. WT females had more PRir on P8 than on P4 ($p < 0.05$), whereas there were no significant effects of age in WT males or ERαKO males and females.

DISCUSSION

This study revealed a dramatic sex difference in the expression of PRir in both the MPN and cVMN of the neonatal WT mouse, with males expressing much

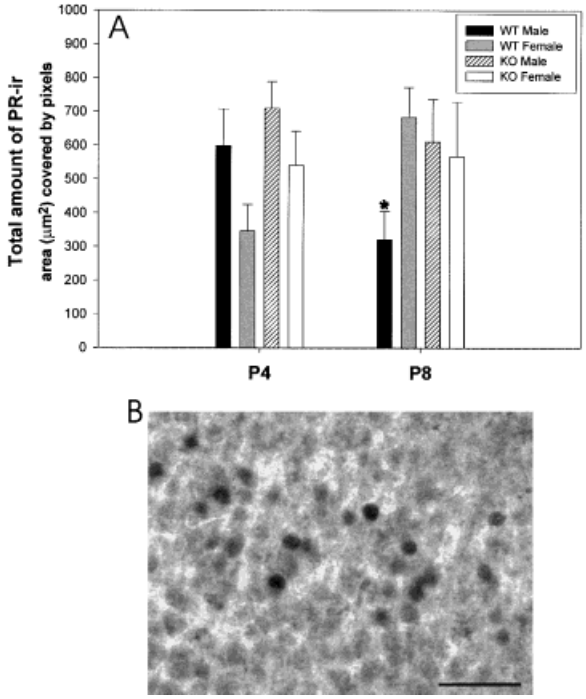

Figure 3 (A) The relative total amount of progesterone receptor immunoreactivity (PRir) in the neocortex on postnatal days (P) 4 and 8 in male and female mice that were either wild-type (WT) or were estrogen receptor α "knock outs" (KO). * significantly different from WT females of the same age. (B) PRir in the neocortex of a P8 female mouse that was WT. Bar = 50 μm.

higher levels of PRir than females. These findings are similar to those reported previously for the developing rat MPN (Wagner et al., 1998a). The absence of PRir in these regions in ERαKO mice, and thereby an absence of the sex difference in PRir, suggests that ERα is a critical component in producing differential sensitivity, between the sexes, to progesterone or its metabolites. The effect of this differential sensitivity during development on sexual differentiation of the brain is unclear, but several reports suggest that neonatal treatment with the PR antagonist RU486 alters the effects of testosterone on the sexual differentiation of MPN structure in rats (Wagner et al., 1999), and modifies subsequent male sexual behavior (van der Schoot and Baumgarten, 1990; Lonstein et al., 2000).

The absence of PR expression in the cVMN or MPN of ERαKO male mice in the present study suggests that endogenous testosterone or its estrogenic metabolites are unable to induce PR expression neonatally in the absence of full length ERα. This contrasts with the ability of exogenous estradiol to induce low levels of PR mRNA and protein expression in the MPN and VMN of ERαKO female mice in adulthood (Shughrue et al., 1997a; Moffatt et al., 1998). This induction has been attributed to the expression of ERβ, the mRNA of which has been detected in both the MPN and VMN of adult ERαKO mice (Shughrue et al., 1997b; Moffatt et al., 1998), as well as to the expression of a splice variant of the disrupted ERα gene. This splice variant, which has been detected in the uterus of ERαKO females, encodes a protein that could potentially bind estradiol (Couse et al., 1995). However, the virtual lack of PRir in the MPN and VMN of neonatal male ERαKO mice demonstrates that full length ERα is necessary for the PR expression in the MPN and cVMN of neonatal mice. Our data do not exclude a role for ERβ in the perinatal induction of PR by estrogenic metabolites of testosterone. In adult rats, ERβ and ERα are coexpressed in individual forebrain neurons (Shughrue et al., 1998). Moreover, ERα and ERβ form heterodimers that bind to DNA consensus sequences of the estrogen response element (Ogawa et al., 1998; Cowley et al., 1997; Pettersson et al., 1997). Because ERβ mRNA is expressed in the hypothalamus/preoptic area of neonatal mice (Karolczak and Beyer, 1998), it may also interact with ERα during development. Therefore, possible effects of ERβ on PR expression during development may depend on expression of the full length ERα. Future studies examining PR expression in neonatal ERβ knockout mice (Krege et al., 1998) could address this issue.

Although the present study does not address the site of action of the ERα gene disruption on PR expression, a direct effect on cells in the MPN and VMN is the most likely mechanism. Estradiol induces PR in these regions in adult and neonatal rats (MacLusky and McEwen, 1978; Brown et al., 1987; Lauber et al., 1991; Shughrue et al., 1997a; Moffatt et al., 1998; Wagner et al., 1998b; Funabashi et al., 2000), and does so specifically in ERα expressing cells (Blaustein and Turcotte, 1989). It is possible that PR expression is reduced in ERαKO males as an indirect consequence of reduced testicular hormone secretion. However, although not examined in neonatal ERαKO mice, circulating testosterone levels in adult ERαKO males are actually elevated compared to wild-type males (Eddy et al., 1996). These findings, taken together, are more consistent with the idea that altered PR expression in the MPN and VMN of ERαKO neonates is due to a lack of estrogen sensitivity in PR expressing cells.

The lack of an effect of the ERα gene disruption on PRir levels in the cerebral cortex demonstrates site specificity in the regulation of PR expression in the MPN and VMN, and is consistent with previous findings demonstrating that progesterone binding in the cortex of the female postnatal mouse brain is not influenced by estradiol (Shughrue et al., 1991a). Levels of PRir were higher in WT females than in WT males on P8. This is consistent with previous findings demonstrating that the number of cells binding a radiolabeled progesterone analogue was higher in the cortex of P8 female mice compared to males (Shughrue et al., 1991b). The consistency of the present data with the progesterone binding data suggests that the sex difference in PRir in mice represents a sex difference in functional progesterone receptors. The absence of a significant effect of ERα disruption suggests that in cortex, activation of ERα alone is not responsible for differential expression of PRir in males and females.

The sex difference in PRir in the neonatal MPN of mice in the present study is similar to that reported for rats (Wagner et al., 1998a), with males expressing high levels of PRir and females expressing very little. However, the sex difference in PRir in the neonatal VMN is quite different between the species. In the VMN of neonatal rats, both sexes express PR, but there are moderately higher levels of PRir in females than in males (Quadros et al., 2000). In contrast, a striking sex difference exists in the VMN of neonatal mice. Males express PR at moderate to high levels, whereas females have little to no PRir in VMN. In addition, prenatal hormone treatment has little effect on PRir levels in the VMN of rats (unpublished observations), whereas the expression of PR is virtually absent in the VMN of ERαKO male mice. This sug-

gests a dependence of PR expression on estradiol in the VMN in mice, and suggests substantial differences between the species in the mechanisms controlling hormonal regulation of PR expression in the developing brain. While the exact nature of these differences remains unknown, species diversity in the ontogeny of the expression of the ER genes, as well as in the possible interactions between ERα and ERβ in the regulation of PR expression, may contribute to species differences in neonatal PR expression.

The present study demonstrates a clear role for ERα in the production of a transient sex difference in PR expression that exists exclusively during development. This suggests that some of the effects of estradiol on the sexual differentiation of specific regions of the brain may be due to the induction of PR via ERα activation.

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