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QUANTITATIVE ESTIMATION OF ESTROGEN AND ANDROGEN RECEPTOR-IMMUNOREACTIVE CELLS IN THE FOREBRAIN OF NEONATALLY ESTROGEN-DEPRIVED MALE RATS

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Abstract—Using quantitative immunocytochemical procedures, the total number of estrogen and androgen receptors was estimated in a large number of hypothalamic and limbic nuclei of male rats, in which brain estrogen formation was inhibited neonatally by treatment with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione. The highest densities of estrogen receptor immunoreactivity were observed in the periventricular preoptic area and the medial preoptic area. Neonatally estrogen-deprived males showed a higher estrogen receptor immunoreactivity than control males in the periventricular preoptic area and the ventrolateral portion of the ventromedial nucleus of the hypothalamus, i.e. those brain areas in which sex differences have been reported, with female rats showing a greater estrogen binding capacity than male rats. The highest densities of androgen receptor immunoreactivity were found in the septohypothalamic nucleus, the medial preoptic area, the posterior division of the bed nucleus of the stria terminalis and the posterodorsal division of the medial amygdaloid nucleus. No significant differences in distribution or total numbers of androgen receptors were found between neonatally estrogen-deprived males and control males.

These findings suggest that neonatal estrogens, derived from the neural aromatization of testosterone, are involved in the sexual differentiation of the estrogen receptor system in the periventricular preoptic area and the ventromedial hypothalamus. The role of neonatal estrogens in the development of the forebrain androgen receptor system is less clear. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: neonatal ATD treatment, androgen receptor, estrogen receptor, estrogens, hypothalamus, preoptic area.

In mammals, testosterone and estradiol derived from neural aromatization of testosterone act synergistically in the developing male brain to organize its structures and functions, i.e. to masculinize and defeminize the neural substrates that later control sexual behavior and neuroendocrine function. In the rat brain, masculinization results in the display of male-typical sexual behavior (mounts, intromissions and ejaculations) in adulthood. Defeminization results in a loss of cyclic release of gonadotropins necessary for ovulation and a loss of female-typical sexual behavior (lordosis, presenting, ear wiggling, hop and dart) in adulthood (e.g., Ref. 37). Thus, gonadally intact male rats display the complete pattern of male coital behavior when pair tested with an estrous female and no female-typical sexual behavior when pair tested with a sexually active male. However, lordosis behavior can be induced in castrated male rats by administering estradiol and progesterone, although the behavior displayed is usually less intense than that of females, and considerably more estradiol is required for its stimulation.[20,42,54]

The development of the female rat brain is generally believed to proceed in the absence of testosterone and estradiol. Gonadally intact female rats display periods of behavioral proceptivity, i.e. seeking out the male partner, and receptivity, i.e. the display of lordosis in response to mounts, around the time of ovulation.[12] However, male-typical sexual behavior, such as intromission-like and ejaculation-like behavior, can be induced in ovariectomized female rats by administering testosterone or estradiol, although

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Abbreviations: AR, androgen receptor; Arc, arcuate nucleus; ATD, 1,4,6-androstatriene-3,17-dione; BNST, bed nucleus of the stria terminalis; DAB, 3,3′-diaminobenzidine tetrahydrochloride; ER, estrogen receptor; IOD, integrated optical density; IR, immunoreactive, immunoreactivity; MePD, posterodorsal medial amygdaloid nucleus; mPOA, medial preoptic area; PVP, periventricular preoptic area; SDN-POA, sexually dimorphic nucleus of the preoptic area; TBS, Tris-buffered saline; VMH, ventromedial nucleus of the hypothalamus.

higher doses are required for its activation than in castrated male rats.[8,11,27,41]

These sex differences in responsiveness to testosterone and estradiol might be influenced by sex differences in the concentration and distribution of androgen and estrogen receptors in the brain. To date, there is evidence indicating that estrogen and androgen receptor concentrations are sexually dimorphic in several areas of the rat brain. Adult female rats have a higher estrogen binding capacity in the periventricular preoptic area (PVP), the medial preoptic area (mPOA), the arcuate nucleus (Arc) and ventromedial nucleus of the hypothalamus (VMH) than adult male rats.[17,18] Sex differences have also been found in basal estrogen receptor (ER) mRNA levels in the ventrolateral portion of the VMH and in the Arc, with female rats showing higher concentrations than male rats.[35] The sex differences in ER biosynthesis are already present early in development.[24,33] Using immunocytochemical procedures, sex differences have been found in both newborn and adult rats, with females showing a higher density of ER immunoreactivity (ER-IR) than males.[57] Sex differences with respect to the androgen receptor (AR) have also been reported: nuclear AR concentrations were found to be higher neonatally in male rats than in female rats; this was most clearly seen in the amygdala.[38] Adult male rats, castrated and treated with physiological doses of testosterone, exhibit significantly higher levels of AR binding compared to similarly treated females in the bed nucleus of the stria terminalis (BNST), PVP and VMH.[47] Presumably, perinatal androgens are involved in the sexual differentiation of brain ER and AR.

We have shown previously that male rats in which brain estrogen formation was inhibited neonatally by administration of the steroidal aromatization blocker 1,4,6-androstatriene-3,17-dione (ATD) can readily show male-typical as well as female-typical sexual behavior (e.g., Refs 3 and 16). In addition, partner preference of ATD males is also incompletely differentiated: when given free access to an estrous female and a sexually active male, ATD males approached both the estrous female to whom they showed male-typical sexual behavior (mounts, intromissions, but rarely ejaculations) and the sexually active male to whom they showed feminine sexual behavior (lordosis, presenting, ear wiggling, hop and dart).[3,6] Furthermore, ATD males showed a response to estrogens which differed from that of control males. After castration and estradiol treatment in adulthood, ATD males displayed high levels of feminine sexual behavior and showed a clear-cut preference for a sexually active male over an estrous female. Similarly treated control males displayed high levels of masculine sexual behavior and showed a clear preference for an estrous female over a sexually active male.[4,5]

In the present study, we evaluated the possible contribution of neonatal estradiol in the sexual differentiation of brain ER and AR using quantitative immunocytochemical techniques.

EXPERIMENTAL PROCEDURES

Animals and treatments

All animal experiments were carried out in accordance with the guidelines of the Animal Facility Department of the Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, which in general follow the NIH guidelines. Male and female Wistar rats (Wu strain, outbred), obtained commercially (Harlan, Zeist, The Netherlands), were housed in single-sex groups of two or three. Food and water were available *ad libitum*. Female rats were time-mated and parturition occurred 22 days later. Within 2–4 h after birth, newborn males received subcutaneously a silastic capsule (SR3: i.d. 1.5 mm, o.d. 2.1 mm, length 5 mm) containing crystalline ATD under ice anesthesia. Control males received empty silastic capsules. The implants were removed at 21 days of age and the animals were housed two to three to a cage of the same treatment. The males were left undisturbed until the age of approximately six months. One week before ER immunocytochemistry, nine ATD and nine control males were castrated under ether anesthesia through a midline abdominal incision.

Immunocytochemistry

Males were anesthetized with sodium pentobarbital (100 mg/rat, i.p.), given an intracardiac injection of heparin (1000 U/rat) and perfused via the aorta with 0.1 M phosphate-buffered saline (pH 7.3), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (300 ml per animal). Brains were removed and postfixed in 4% paraformaldehyde for 2 h and placed in 0.1 M phosphate-buffered saline. Sections of 50 μm were cut on a Vibratome using ice-cold Tris-buffered saline (TBS; 0.05 M, 0.9% NaCl, pH 7.6). TBS was used to rinse the sections for at least 1 h (three times) between all steps of the immunocytochemical procedure. All antibodies were diluted in 0.05 M TBS containing 0.5 M NaCl and 0.5% Triton X-100 (pH 7.6). For ER-IR, the antibody ER-21 (gift of G. Greene, University of Chicago), which is a rabbit polyclonal antibody directed against the N-terminus (amino acids 1–21), i.e. the estrogen binding domain of the ER, was used (1 μg/ml). ER-21 has been validated previously.[14,15,39] For AR-IR, the antibody PG-21 was used (gift of G. Greene, University of Chicago), which is a rabbit polyclonal antibody directed against a synthetic peptide corresponding to the first 21 amino acids of the rat AR (2 μg/ml; e.g., Ref. 44). PG-21 has been validated extensively (e.g., Refs 19, 55 and 56).

Free-floating sections were incubated with primary antiserum (ER-21 or PG-21) and left for 1 h at room temperature and subsequently at 4°C overnight. Goat anti-rabbit immunoglobulin (Betsy, 1:100; Netherlands Institute for Brain Research) was applied for 1 h at room temperature followed (after rinsing with TBS) by peroxidase–antiperoxidase (1:1000; Netherlands Institute for Brain Research) for 1 h. All incubations were done on a rocking table. As chromogen, 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.5 mg/ml) with 0.01% H_2O_2 and 0.2% nickel ammonium sulfate in 0.05 M TBS (pH 7.6) was used. Sections were then mounted on chrome–alum-coated slides, dehydrated and coverslipped using entellan.

Quantitative analysis

The densitometrical analyses were performed on an IBAS-KAT image analysis system (Kontron). The image

analyser was connected to a Bosch TYK9B TV camera equipped with a chalnycon tube mounted on a Zeiss microscope. The microscope was equipped with a planapo objective, a light source connected to a stabilized power supply and a scanning stage under control of both a joystick and the image analyser. All measurements were done using a 560-nm small band filter (Schott), which coincides with the absorption maximum of the DAB–nickel precipitate in the sections.

For each section, the analysis consisted of the following steps: (i) loading of an image containing no immunostaining at a $\times 10$ magnification for measurement of the background; (ii) loading of a $\times 2.5$ magnification image covering that part of the section which contained the area(s) of interest; (iii) loading of 12 (4×3) 768×512 images with $\times 10$ magnification; (iv) manual outlining of the measuring areas (anatomical structures) in the reconstituted $\times 2.5$ magnification image. With exception of the sexually dimorphic nucleus of the preoptic area (SDN-POA), the outlines of the measuring areas were based on the immunocytochemical staining. For the sexually dimorphic nucleus, some sections were counterstained with Cresyl Violet in order to delineate more faithfully the SDN-POA boundaries; (v) feeding in the computer the names and the left/right side dimensions of the outlined structures; (vi) calculation of a mask for the immunoreactive cells in each outlined structure and measurement of the total area of that mask in each outlined structure (area mask); (vii) measurement of the area of the outlined structure (area field); (viii) calculation of the optical density of the area mask (OD_{mask}), which measures the concentration of DAB in the outlined area ($mg/\mu m^3$); (ix) calculation of the integrated optical density (IOD; $IOD_{mask}=OD_{mask} \times$ area mask). In this way, the total amount of DAB, which is an estimate for the total number of ER- or AR-IR nuclei, was measured in each outlined area. The data obtained were first analysed for normal distribution using the Kolmogorov–Smirnov test and the group means for each area of interest were subsequently subjected to Student's *t*-test. The $P=0.05$ level was used as the upper limit for statistical significance.

RESULTS

Estrogen receptor immunoreactivity

In both ATD and control males, dense populations of ER-IR cell nuclei were observed in the PVP, mPOA, BNST, specifically in the anterior and posterior medial divisions, Arc, ventrolateral portion of the VMH and in the posterodorsal medial amygdaloid nucleus (MePD). Furthermore, the PVP and mPOA contained the greatest numbers of ERs. Examples of ER-IR cell nuclei in the PVP, mPOA and VMH are shown in Fig. 1. Analysis with Student's *t*-test showed that the total number of ER-IR nuclei, i.e. the IOD, of ATD males was significantly higher compared to control males in the PVP ($t=3.39$, d.f.=16, $P=0.01$) and in the VMH ($t=2.42$, d.f.=15, $P=0.05$) (Table 1). ATD males had similar densities of ERs as control males in the mPOA, BNST, Arc and MePD (Table 1). Analyses with Student's *t*-test revealed that the total area occupied by ER-IR cells, i.e. the area mask, was significantly higher in the PVP ($t=4.8$, d.f.=16, $P<0.001$), the mPOA ($t=2.6$, d.f.=16, $P=0.02$) and the VMH ($t=3.6$, d.f.=15, $P=0.01$) of ATD males compared to control males (Table 1).

Androgen receptor immunoreactivity

In both ATD and control males, AR-IR cell nuclei were found in the septohypothalamic nucleus, the mPOA, the SDN-POA, the anterior and posterior divisions of the BNST, the Arc, the VMH and through the rostrocaudal extent of the medial amygdaloid nucleus, i.e. anterior, anteroventral, anterodorsal, posteroventral and MePD (see Table 2). Furthermore, the greatest numbers of ARs were observed in the septohypothalamic nucleus, the mPOA, the posterior division of the BNST, and the MePD. Examples of AR-IR cell nuclei in the mPOA containing the SDN-POA, the posterior division of the BNST, and posterior medial amygdaloid nucleus are presented in Fig. 2. Analysis with Student's *t*-test revealed that the total amount of AR-IR nuclei, i.e. the IOD, was similar in ATD and control males for each area measured (Table 2). Also, no differences between ATD and control males were found in the total area occupied by AR-IR cells, i.e. the area mask (Table 2).

DISCUSSION

Estrogen receptor immunoreactivity

Neonatal inhibition of brain estrogen biosynthesis in male rats increased ER levels in the PVP, an area known to be involved in gonadotropin release, and the ventrolateral portion of the VMH. As described in the Introduction, sex differences have been reported in the PVP and VMH, with female rats showing a higher estrogen binding capacity[18,33] or a higher ER-IR[57] than male rats. Unfortunately, no female controls were used in the present study. Therefore, it is not known how closely the ER levels found in the PVP and VMH of neonatally ATD-treated males approximate to those found in normal females. It could be possible that neonatally ATD-treated males are intermediate between normal males and females with respect to their ER levels, as was found earlier with respect to their partner preference.[4] This would suggest that either androgens or prenatal estrogens are also involved in the sexual differentiation of ER levels in the PVP and VMH. Some supportive evidence for a possible contribution of prenatal androgens or estrogens to the sexual differentiation of ER levels includes the finding that neonatal castration of male rats did not entirely feminize their ER levels in the VMH.[34] On the other hand, neonatal castration of male rats increased ER levels in the PVP, Arc and mPOA to levels indistinguishable from those found in normal females,[34] suggesting that the sexual differentiation of brain ER takes place primarily neonatally under the influence of androgens or estrogens.

Neonatal inhibition of brain estrogen biosynthesis did not affect ER levels in the Arc and mPOA, although the total area occupied by ER-IR cells in the mPOA was higher in neonatally ATD-treated

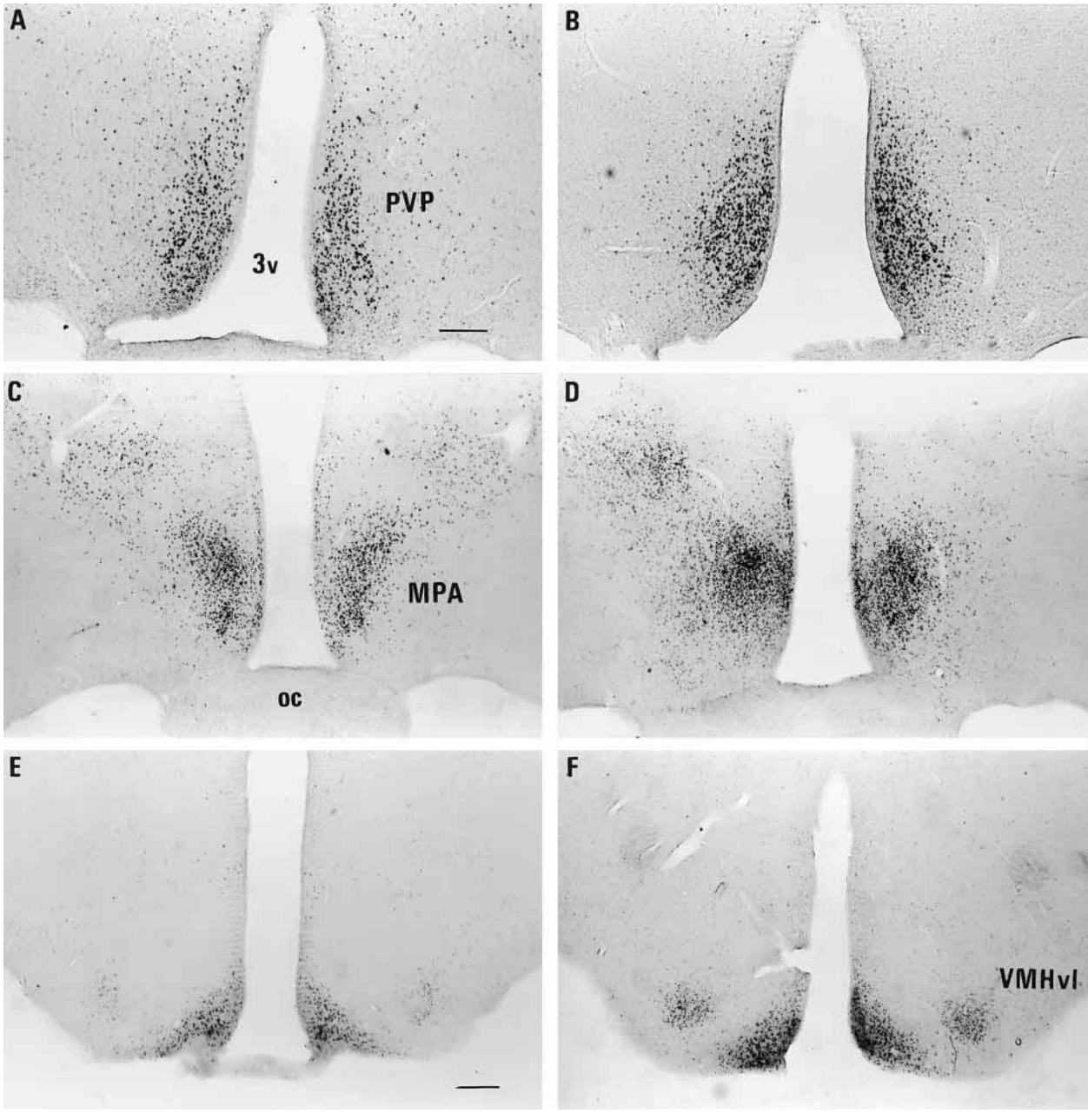

Fig. 1. Photomicrographs showing ER-IR in neonatally ATD-treated male and control male rats. (A, B) The PVP of a control male (A) and of an ATD male (B). (C, D) The mPOA of a control male (C) and of an ATD male (D). (E, F) The VMH of a control male (E) and of an ATD male (F). Scales: 1 cm=195 μm (A, B); 1 cm=310 μm (C–F).

males than in control males (see Fig. 1). Kühnemann *et al.*[33,34] reported that castration immediately after birth increased ER levels in the Arc and mPOA, whereas castration one day after birth failed to do so. Presumably, the sex differences in brain estrogen levels in the mPOA and Arc might represent an extremely rapid response to the hypothalamic surge in estradiol occurring in males between 0 h *in utero* and 1 h after delivery.[46] Since ATD treatment was not initiated until 2–4 h after birth, ATD males were most likely exposed to this hypothalamic surge in estradiol. Therefore, ATD males' brains could already have been partly organized in a male direction, i.e. ER levels were already lowered partly in the mPOA and almost completely in the Arc. In support of the role of neonatal estrogen in the sexual differentiation of ER in the preoptic area are the findings of DonCarlos *et al.*[25] Female rats neonatally treated with the synthetic estrogen, diethylstilbestrol, showed at the age of 28 days reduced ER mRNA levels in the

Table 1. Estrogen receptor immunoreactivity of neonatally 1,4,6-androstatriene-3,17-dione-treated male and control male rats in various hypothalamic and limbic nuclei

Region of labeled cells	Total number of ERs ($\times 10^3$)†		Area occupied by ERs (μm^3, $\times 10^3$)	
	Control	ATD	Control	ATD
Periventricular preoptic area	10 ± 0.9	15 ± 1.1*	21 ± 0.8	28 ± 1.2*
Medial preoptic area	21 ± 1.9	24 ± 1.4	40 ± 1.8	48 ± 2.2*
Bed nucleus of the stria terminalis				
Anterior	5 ± 0.8	5 ± 0.6	16 ± 1.5	19 ± 1.7
Posterior	7 ± 1.2	8 ± 1.2	22 ± 2.2	25 ± 2.5
Arcuate nucleus	7 ± 0.5	7 ± 0.5	13 ± 0.7	13 ± 0.7
Ventromedial hypothalamus	1.9 ± 0.3	2.8 ± 0.2*	5 ± 0.6	8 ± 0.5*
Medial amygdaloid nucleus				
Posterodorsal	8 ± 0.9	9 ± 0.9	25 ± 2	26 ± 1.8

†The total number of ER-IR nuclei is estimated by taking the product of the optical density, which measures the concentration of the chromogen DAB in each outlined area, and the area occupied by ERs.
*Significantly ($P<0.05$) higher compared to control males.
Data are expressed as mean ± S.E.

Table 2. Androgen receptor immunoreactivity of neonatally 1,4,6-androstatriene-3,17-dione-treated male and control male rats in various limbic and hypothalamic nuclei

Region of labeled cells	Total number of ARs ($\times 10^3$)†		Area occupied by ARs (μm^3, $\times 10^3$)	
	Control	ATD	Control	ATD
Septohypothalamic nucleus	17 ± 2.4	17 ± 2.9	51 ± 5.3	50 ± 7.0
Medial preoptic area	16 ± 1.2	17 ± 1.6	50 ± 3.0	50 ± 3.0
Sexually dimorphic nucleus	6 ± 0.5	5 ± 0.6	13 ± 1.0	11 ± 0.8
Bed nucleus of the stria terminalis				
Anterior	10 ± 0.7	10 ± 1.0	40 ± 2.2	38 ± 3.0
Posterior	22 ± 2.3	25 ± 4.2	71 ± 5.3	77 ± 9.0
Arcuate nucleus	2 ± 0.2	3 ± 0.4	6 ± 0.3	7 ± 0.8
Ventromedial hypothalamus	11 ± 0.7	11 ± 1.4	41 ± 2.3	41 ± 3.9
Medial amygdaloid nucleus				
Anterior	4 ± 0.5	5 ± 0.8	20 ± 2.3	25 ± 3.3
Anterodorsal	6 ± 0.6	5 ± 0.7	27 ± 2.2	23 ± 2.5
Anteroventral	4 ± 0.4	3 ± 0.3	13 ± 1.0	12 ± 1.0
Posterodorsal	19 ± 1.8	19 ± 3.3	64 ± 4.7	57 ± 6.0
Posteroventral	4 ± 0.4	4 ± 0.7	15 ± 1.4	16 ± 2.1

†The total number of AR-IR nuclei is estimated by taking the product of the optical density, which measures the concentration of the chromogen DAB in each outlined area, and the area occupied by ARs.
Data are expressed as mean ± S.E.

preoptic area, comparable to the levels seen in intact males. The non-aromatizable androgen, dihydrotestosterone, had no effect on ER mRNA in the preoptic area of females.

The sex differences in ER levels are presumably established within 24 h after birth for the PVP and mPOA, whereas the sex difference in the VMH emerges later, between five and 10 days after birth.[33] Thus, sex differences in ER levels in different brain areas seem to be expressed asynchronously, providing a possible mechanism for variation in the duration of critical periods for androgen- or estrogen-mediated sexual differentiation of specific neural functions. For instance, masculinization and defeminization of sexual behavior are probably two separable processes, which occur at slightly different times during development (e.g., Refs 7, 13 and 52). Our behavioral data suggest that defeminization of the male rat brain takes place primarily neonatally under the action of estradiol and that masculinization of the brain occurs mainly both prenatally and immediately after birth under the synergistic action of testosterone and estradiol.

The sex differences in ER levels in the PVP, mPOA, Arc and VMH may contribute to the regulation of sex-specific estrogen-dependent functions. For instance, female rats are capable of showing a preovulatory luteinizing hormone surge after adult gonadectomy and treatment with a large dose of estradiol, whereas male rats are not.[40] Neonatal castration makes it possible for males to show a preovulatory luteinizing hormone surge in response to estrogen, whereas neonatal treatment of female rats with testosterone eliminates this capacity to exhibit estrogen-induced surges (for overview see Ref. 26). Whether functional responses, such as an estrogen-induced

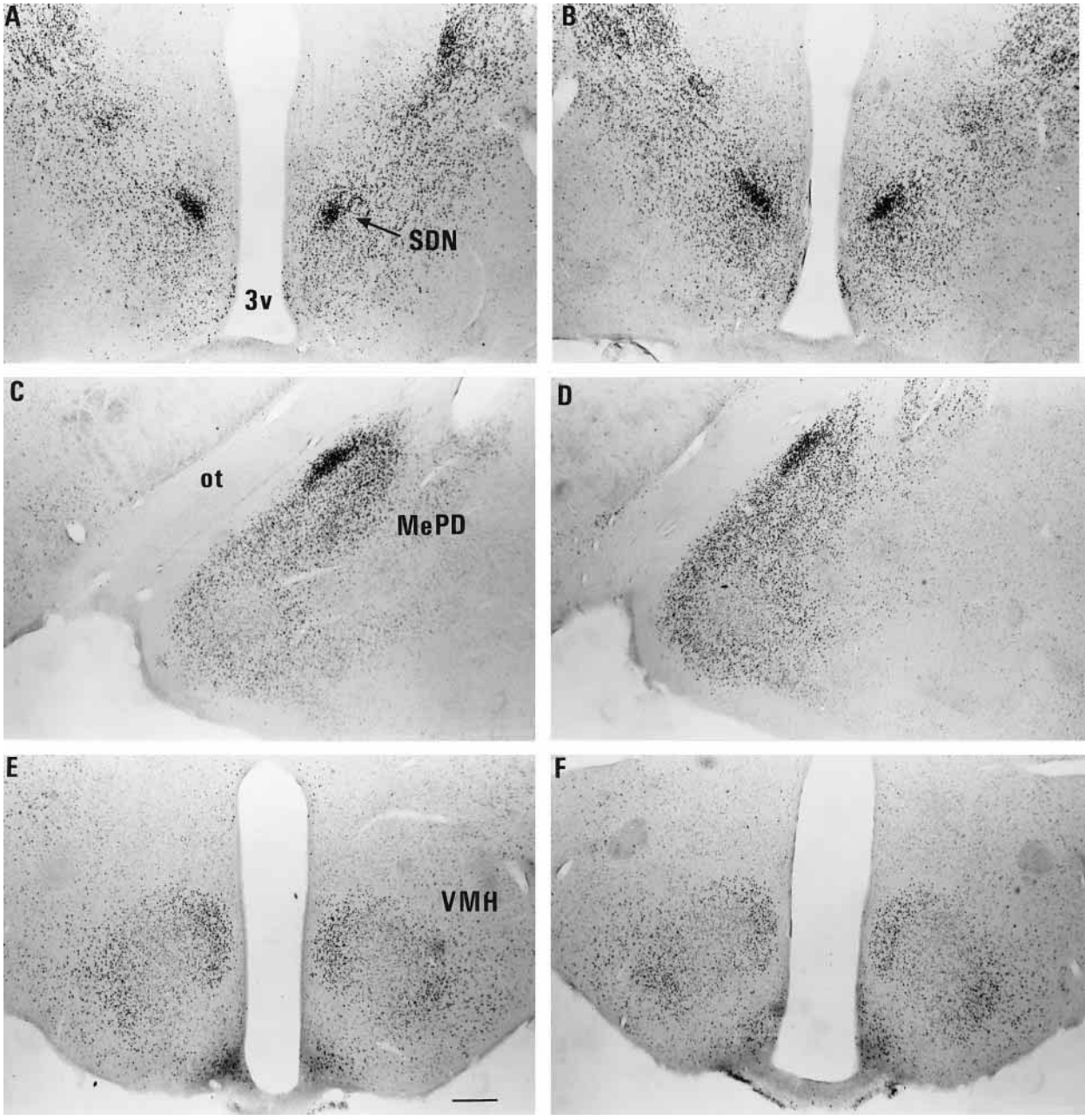

Fig. 2. Photomicrographs (1 cm=310 μm) showing AR-IR of neonatally ATD-treated male and control male rats. (A, B) The mPOA including the sexually dimorphic nucleus of a control male (A) and of an ATD male (B). (C, D) The MePD of a control male (**C**) and of an ATD male (D). (E, F) The VMH of a control male (E) and of an ATD male (F).

luteinizing hormone surge, are directly proportional to the number of ER-containing neurons in a particular brain area remains to be investigated. Interestingly, a co-existence of ER and neurotensin has been found in the PVP.[2] Neurotensin appears to be involved as an interneuron in the regulation of the release of luteinizing-releasing hormone (e.g., Ref. 1).

For activation of feminine sexual behavior in the male rat, a much higher dose of estradiol is required than in the female rat.[20,28,42,54] This is confirmed by previous behavioral findings in our laboratory.[4,5] Neonatally ATD-treated male rats, castrated in adulthood and subsequently treated with estradiol, show high levels of feminine sexual behavior and a partner preference for a sexually active male, whereas similarly treated control males show high levels of masculine sexual behavior and a partner preference for an estrous female. Only when treated with extremely high doses of estradiol (blood serum levels

1000 pmol/l; normal blood serum levels ≈7 pmol/l), normal males show feminine sexual behaviors (unpublished observations in our laboratory). These findings suggest that the reduced capacity to respond to estrogen in adulthood is associated with lower levels of ER in the male rat brain.

Meredith *et al.*[39] reported that the ER-21 antiserum is able to detect both occupied and unoccupied ERs in the guinea-pig, since ER-21 immunostaining was not affected by a high dose of 17β-estradiol (50 μg). However, in a pilot study, we found a more intense staining with the ER-21 antiserum after the male rats were castrated. It has been reported that castration leads to an up-regulation of ER mRNA,[49] whereas acute estradiol treatment down-regulates ER mRNA.[35] In the present study, castration has presumably led to an up-regulation of ER in both ATD and control males, although the possibility cannot be ruled out that the ER-21 antiserum could be more efficient at detecting unoccupied ERs in the rat brain as opposed to the guinea-pig brain. If ER levels were up-regulated by castration, then the ER levels in the present study represent the maximal capacity rather than physiological levels.

Androgen receptor immunoreactivity

Neonatal inhibition of brain estrogen biosynthesis in male rats did not affect forebrain AR levels. This suggests that neonatal estrogens do not play a significant role in the sexual differentiation of the forebrain AR system. However, it is not clear to what extent ARs are sexually differentiated, i.e. sexually dimorphic, in adulthood. Reports on sex differences in androgen binding capacity have been conflicting. Biochemical assays of androgen binding in whole preoptic area and/or hypothalamus dissections have not revealed sex differences.[29,48] However, measuring AR binding in more discrete nuclei within the preoptic area and hypothalamus has demonstrated sex differences.[47] In particular, the caudal part of the mPOA showed sex differences, with males having more testosterone target neurons that project to the midbrain than females.[36] This might not be proof for sex differences in ARs, since testosterone can be aromatized to estradiol within the CNS. Therefore, a labeled cell following an injection of radioactively labeled testosterone may represent estrogen binding to ERs. Sex differences in AR levels have been found during the first 10 days of life, especially in the amygdala.[38] In this latter study, however, males and females were left gonadally intact, which makes it very likely that the sex differences in AR levels reflect sex differences in endogenous testosterone or dihydrotestosterone levels. Presumably, there are no large sex differences in the distributions and densities of AR-containing cells, although small, subtle quantitative differences in certain regions of the male and female rat brain cannot be ruled out. It seems unlikely that possible sex differences in AR levels are caused by the actions of estrogen during early development, since neonatally ATD-treated males showed similar distributions and densities of AR-IR as control males. However, no female controls were used in the present study, so it is not known whether or not any sex differences in AR levels would have been found using the assay conditions of the present study. Therefore, we cannot determine from the present data whether or not sexual differentiation of forebrain ARs occurs and, if it happens, whether it takes place pre- or neonatally under the influence of androgens or estrogens.[9,10,43,45,51,53] Future studies should address these questions.

Interestingly, a dense cluster of AR-IR was found in the SDN-POA (see Fig. 2), as was reported earlier for AR mRNA by Simerly *et al.*[50] This was not seen with ER-IR. In the adult rat, the volume of the SDN-POA is determined by the perinatal action of androgens.[21,22,23,30,32] Jacobson *et al.*[31] have found that gonadectomized and adrenalectomized male rats showed a higher percentage of radioactively labeled cells in the SDN-POA following exposure to [^{3}H]testosterone than gonadectomized and adrenalectomized females.[31] This sex difference might be due to a difference in AR levels in the mPOA.[29] In the present study, no effect of neonatal ATD treatment was found on the number of ARs in the SDN-POA or on the SDN-POA occupied by ARs. We expect that the volume of the SDN-POA of neonatally ATD-treated males is smaller than that of control males, since perinatal ATD treatment to male rats reduced SDN-POA volume in adulthood.[30] Presumably, the absence of changes in the number of ARs in the SDN-POA or in the SDN-POA occupied by ARs suggests that there are either no changes in the size of the SDN-POA or that AR immunostaining in the SDN-POA has increased with ATD treatment to compensate for the smaller size. We are currently studying the SDN-POA volume in neonatally ATD-treated male rats.

It has been suggested that the binding of the PG-21 antiserum to androgen receptors requires occupation of the receptor. Zhou *et al.*[58] reported that testosterone withdrawal by castration eliminated all nuclear staining, whereas dihydrotestosterone or testosterone treatment 15 min before perfusion restored nuclear staining. In the present study, the ATD and control males were left gonadally intact for the AR immunocytochemistry. We do not believe that the number of occupied ARs differs between neonatally ATD-treated males and control males, since neonatally ATD-treated males and control males have similar levels of endogenous testosterone.[6]

CONCLUSIONS

The available evidence points to sex differences in ER levels in the PVP, mPOA, Arc and VMH. The development of these sex differences in forebrain ER levels seems to be partly dependent on the neonatal

action of estrogens, derived from the neural aromatization of testosterone. To date, it is not clear whether or not sexual differentiation of forebrain ARs occurs and, if so, whether there is any contribution of neonatal estrogens to the development of the AR system.

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