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Steroid Control of Higher Brain Functions

GENE EXPRESSION FOR ESTROGEN AND PROGESTERONE RECEPTOR mRNAS IN RAT BRAIN AND POSSIBLE RELATIONS TO SEXUALLY DIMORPHIC FUNCTIONS

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Summary—A clear neuroendocrine sex difference lies in the ability of the female rat to produce an ovulatory surge of luteinizing hormone. Preoptic neurons, as they respond to estrogen and progesterone, have been proven to be involved in this mechanism, with an emphasis on the possible participation of neurons in the anteroventral periventricular nucleus and the suprachiasmatic portion of the preoptic area (POA). Further, prominent morphological sex differences have been reported in the rat medial POA. To examine expression of the estrogen receptor (ER) and the progesterone receptor (PR) messenger RNAs (mRNAs) in these critical preoptic neurons, we have used *in situ* hybridization with tritiated single-stranded DNA probes complimentary for ER and PR mRNA. ER mRNA containing cells were found in the periventricular, suprachiasmatic and medical preoptic cell groups, in a manner which agrees with steroid hormone autoradiography. In the female rat, preoptic neurons expressing PR mRNA were distributed very similarly to those for ER mRNA. Moreover, in the male rat brain, all subsets of preoptic neurons which express the PR gene in the female were also detected in the male. Thus, the distribution of PR expressing cells was very similar between females and males. We conclude that the insensitivity to the male to progesterone, as regards the hormonal control of ovulation, cannot be due to a total failure of PR gene expression in a specific subset of POA neurons. Instead, male preoptic neurons must be less sensitive to neural or hormonal inducers in the physiological range or perhaps lack sufficient levels of a transcription factor linking progesterone responsive elements to the start sites of hormone-controlled genes.

INTRODUCTION

Discovery of exact locations of sex steroid receptors in the brain [1] opened the way for neuroendocrine analyses of brain functions which depend on classical nuclear receptors. Indeed, a neural circuit determined for homone-dependent female rat reproductive behavior [2, 3] now constitutes an "expression system" for genes influenced by estradiol and progesterone, and involved in that behavior. For example, the mRNA for preproenkephalin, as expressed specifically in the ventromedial hypothalamus [4], can be correlated tightly with lordosis behavior.

Gene expression for the nuclear hormone receptors themselves now provides a novel route of investigation of hormone effects on brain function. Steroid hormone receptors are products of a gene superfamily [5] which can act as transcription factors [6]. Mechanisms by which these proteins affect the initiation or efficiency of transcription are under intense investigation [7]. Steroid receptors are expressed in neurons, as revealed with steroid autoradiography [1] and show great regional specificity in brain tissue. Ligand binding activates these transaction factors, resulting in alterations in neuronal communication which can culminate in changes in behavior.

Sex differences

Sex differences in hormone binding [8–12] and receptor regulation [13–15] have been reported for steriod receptors in some brain regions. However, the differences could arise from post-translational modifications including phosphorylation [16, 17] and affinity for 90K heat

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shock protein [18] which affect the ability of the receptor to bind ligand. Therefore, we have begun investigations of sex differences and regulation of the gene products which precede translation of the estrogen and progestin receptor proteins. So far, *in situ* hybridization has been the most successful technique for measuring the rare estrogen receptor (ER) and progestin receptor (PR) messages in the brain.

In situ hybridization has proven successful for the detection and quantification of ER and PR messenger RNA (mRNA) in the medial basal hypothalamus [19, 20]. We have found that estrogen down-regulates (Fig. 1) the level of mRNA for its own receptor [19] in the ventromedial nucleus and arcuate nucleus of the hypothalamus. Moreover, the regulation of ER mRNA level by estradiol is sexually differentiated: it is robust in genetic females but not males [22].

The regulation of the mRNA for the PR in the hypothalamus is quite different from ER. Estrogen increases the number of neurons expressing the PR gene and produces an approx. 3.5-fold increase in message level in cells of the ventromedial nucleus and arcuate nucleus, but not the amygdala of the female rat [20, 21].

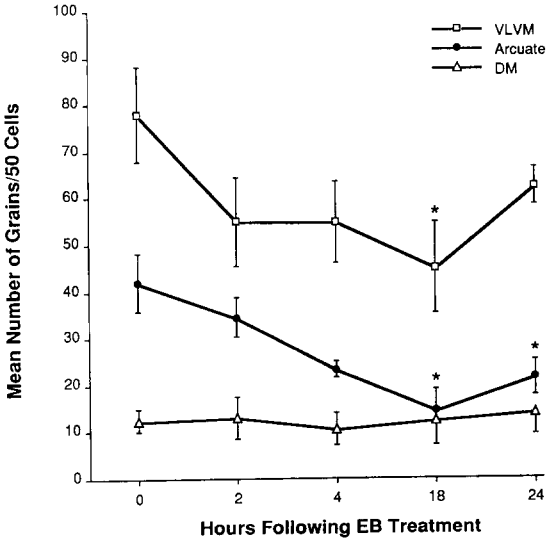

Fig. 1. Shown here are the effects of EB on the level of ER mRNA in the ventrolateral aspect of the ventromedial (VLVM), arcuate and dorsomedial (DM) nuclei. The means and SEMs represent the average number of grains over a sample of 50 cells/brain region from ($n = 4$/group) ovx female rats administered the sesame oil vehicle (0) or 10 μg EB 2, 4, 18 or 24 h prior to sacrifice. *Indicates that these values are decreased significantly compared to 0 control as determined by one-way analysis of variance using Dunnett 2-tailed *post-hoc* comparisons. This figure is taken from Lauber *et al.* [19] where methods and quantification of the ER mRNA message are described in detail.

Here, estrogen regulation of PR mRNA level corresponds to estrogen induction of the receptor [23, 24]. The role of the PR in the ventromedial nucleus has been considered in relation to female reproductive behavior, and with regard to apparent sex differences both in PR induction and the behavior [25–28]. Thus it is interesting that estrogen treatment increases PR mRNA levels in females (Fig. 2) but not males [21]. In summary, these findings on PR gene expression relate the synthesis of a transcription factor to a specific behavior for the first time: up-regulation of the PR mRNA in a sex-specific way is related, in turn, by each of these characteristics to the production of a specific reproductive behavior (lordosis).

The ability to produce an ovulatory surge of luteinizing hormone (LH) is clearly a sexually differentiated estrogen- and progesterone-dependent neuroendocrine function. Sex steroid hormones act on preoptic neurons to promote the ovulatory LH surge [29] but not on the LHRH-containing neurons directly, as these cells do not concentrate estrogen [30] or progesterone [31]. It is not yet clear exactly which preoptic neurons control ovulation. Although a morphological sexual difference in the preoptic area (POA) would implicate neurons in this region [32], very small medial preoptic lesions centred on the suprachiasmatic region of the POA are able to reduce or block estrus cycling and LH surges [33–36]. Based upon these observations and the distribution of estrogen and progestin binding sites in the POA [24, 37, 38] we hypothesized, that both the suprachiasmatic POA and the sexually dimorphic cell group in the POA would contain neurons expressing the gene for the ER and the PR. Further, since males do not respond to estrogen and progesterone with an LH surge, or with levels of lordosis equivalent to females [39–41], we wanted to determine if cells expressing PR mRNA might be distributed differently in males and females.

METHODS

Adult Sprague–Dawley rats (300–350 g) gonadectomized by the supplier (Charles Rivers, Wilmington, MA), or intact, were housed on a 12:12 light/dark cycle with constant access to food and water. Sesame oil (vehicle control) estradiol benzoate (EB) (10 μg; s.c.) or testosterone (1 mg, s.c.) was administered 24 h prior to sacrifice by decapitation. The

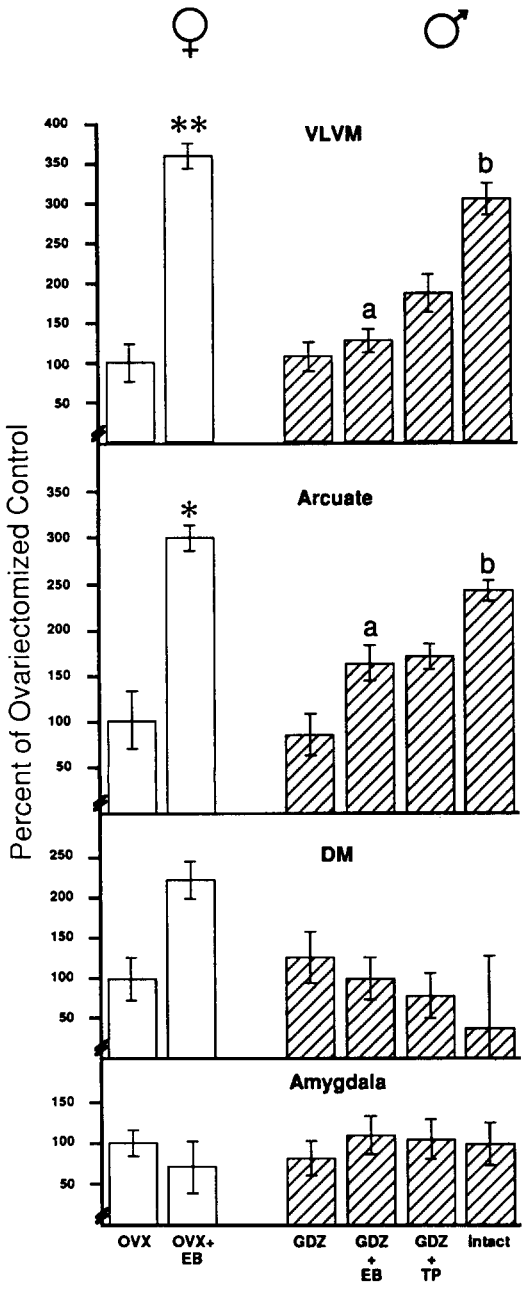

Fig. 2. The effects of hormone treatment on PR mRNA in the VLVM, arcuate, DM and amygdaloid nuclei from female and male rats. Ovx females and gonadectomized (Gdz) males ($n = 4$/group) were administered sesame oil vehicle, 10 μg EB or 1 mg TP 24 h prior to sacrifice. Some males remained gonadally intact. Shown here are the mean numbers of grains per 50 cell sample ($\pm$SEM) expressed as percent of ovx control for the respective brain regions. One-way analysis of variances followed by *post-hoc* comparisons were used to determine if the effects of hormonal treatment were significant. This figure is taken from Lauber *et al.* [21] where methods and quantification of the PR mRNA message are described in detail. $^{**}P < 0.01$, compared to ovx female; $^{*}P < 0.05$, compared to ovx female; $^{a}P < 0.05$ compared to EB female (*post-hoc* 2-tailed *t*-test); $^{b}P < 0.05$, compared to ovx female (*post-hoc* 2-tailed *t*-test).

brains were removed, blocked in a Jacobowitz brain slicer, cryostat sectioned (10 μ) and postfixed in paraformaldehyde as reported previously [19, 20, 42].

Probe preparation

The probes were [^{3}H]single-stranded DNAs prepared from the full-length open reading frame of the human ER cDNA or from the human progesterone cDNA fragment (clones received from Dr P. Chambon).

E. coli cells were transformed with the cDNA in plasmids and amplified large scale cultures were grown. Plasmids were purified using an alkaline lysis procedure and separated from chromosomal DNA using pZ523 columns and accompanying protocol (5 Prime 3 Prime Inc., Paoli, PA). Endonuclease restriction enzymes were used to linearize plasmid DNA prior to *in vitro* transcription of message-sense RNA transcripts. The RNA templates were combined with DNA, random primers (Boehringer Mannheim, GmbH), all 4 tritiated dioxynucleotides (Amersham, Arlington Heights, IL), reverse transcriptase (Boehringer Mannheim) and buffer and incubated overnight at 37°C. The RNA transcript was hydrolyzed, and preparations were run through G-25 sephedex spin columns, yielding high sp. act. (1×10^8 cpm/μg) single-stranded DNA probes free of unincorporated nucleotides. Evaluation of the DNA using alkaline agarose gel electrophoresis [43] revealed that these probes consisted of radiolabeled fragments ranging from approx. 50–500 nucleotides in length.

As controls, the same purified plasmids were used except they were linearized with different endonucleases. The RNA transcription was initiated at the opposite promoter from above, thereby yielding the antisense-message template, used in the reverse transcription reactions as above. These preparations resulted in [^{3}H]sense-message probes which do not hybridize to the mRNA in the tissue. This configuration results in a good control probe since the same relative concentration of each nucleotide is maintained, although the probe does not hybridize specifically to the tissue [19–21].

Another control procedure used was to treat the tissue with RNase A prior to hydribrization with the [^{3}H]antisense-message probes [19–21]. No specific hybridization was detected in RNase treated sections.

The methods and buffers used in the *in situ* hybridization have been described

previously [42]. Briefly, sections were covered with 20 μl prehybridization buffer and incubated at 37° C for 2 h in air-tight humidified plastic boxes. Slides were rinsed in 2 × SSC followed by an ETOH rinse and desiccation prior to hybridization. Each section received 70,000 cpm of heat-denatured [^{3}H] probe in 20 μl of hybridization buffer. Hybridization was conducted for 72 h at 37° C. Sections were rinsed in 2 × SSC plus 0.05% sodium pyrophosphate buffer for a total of 20 min, 0.5 × SSC with 0.05% sodium pyrophosphate for 48 h, then dehydrated in a series of alcohols (70, 90 and 100%) diluted with 0.3 ammonium acetate (pH 5.5). The slides were dried overnight at room temperature, dipped in Kodak NTB 2 emulsion, then desiccated and stored in the dark at 4°C for 4–8 months. The slides were developed using Kodak D19 developer and Kodak fixer, stained with cresyl violet, dehydrated in alcohols, cleared with Histoclear (National Diagnostics, Somerville, NJ) and coverslipped with permount.

Charting the distribution of labeled cells

The preoptic tissue used here was taken from the same animals used for quantitative studies on ER and PR gene expression in the mediobasal hypothalamus. The ER mRNA study was comprised of a total of 20 ovariectomized (ovx) rats ($n = 4$ oil-treated; $n = 16$ received 10 μg EB 2, 6, 18 or 24 h prior to sacrifice). For PR mRNA a total of 24 ovx rats were used [$n = 4$ vehicle treated, $n = 4$ ovx females given 10 μg EB, $n = 12$ castrated males which were given oil, 1 mg testosterone propionate (TP) or 10 μg EB and $n = 4$ intact males]. During the course of the quantitative studies on steroid receptor gene expression at the level of the ventromedial and arcuate hypothalamic nuclei [19, 21], we collected enough preoptic tissue for detecting and charting ER and PR-expressing neurons, but not enough for the type of quantitative analyses done on those studies. Here, for PR mRNA, we systematically examined 6 female rats' preoptic tissue (3 ovx controls and 3 EB-treated for 24 h), and 10 male rats' preoptic tissue (3 castrated controls, 4 treated with TP and 3 intact males). For ER mRNA we systemically examined preoptic tissue from 9 female rats (4 ovx controls and 5 EB-treated).

All sections through preoptic tissue hybridized with the [^{3}H]single-stranded DNA probes for the ER and PR mRNAs and were examined systematically with a light microscope for the detection of neurons expressing ER and PR messages, respectively. Cells in the POA which were mapped had gains $\geqslant 3$ times the average number of grains overlying cells in the lateral hypothalamus (of the same section), which is devoid of ERs and PRs. Specific hybridization for these particular ER and PR probes had already been established quantitatively and was evident in the ventromedial and arcuate nuclei, but not in the thalamus or cortex of this same tissue [19–21].

Charts were constructed by tracing outlines of sections which had the greatest number of labeled cells, and scanning those sections systematically, plotting 1 dot per labeled cell. For convenience of illustration, all cells are recorded as being on 1 side of the brain, with the other side of the drawing saved for abbreviations. Then, for each group, 2 other sections also among the highest in terms of numbers of labeled cells, were charted onto the same drawing. This yields a number of dots per cell group equal to 6 times the average number in the best labeled sections, and helps to prevent false assignment of differences derived from peculiarities of individual sections. In the female, the neurons best labeled for ER mRNA were usually in the ovx control group; while for PR, they were from the EB-treated animals. For males, the greatest numbers of cells labeling for PR mRNA were among those animals which were castrated and treated with TP or those which remained intact. Thus, we were assessing the capacities of preoptic neurons to express ER or PR mRNA under the best hormone condition for each sex.

RESULTS

In situ hybridization with [^{3}H]single-stranded DNA probes for the ER and PR messages revealed labeled cells in the POA. As these mRNAs are rare, numbers of grains per cell were smaller than has been typical for mRNAs for neuropeptides [44, 45], but were clearly above background [Fig. 3(A) and (C)]. For both ER and PR mRNAs neurons were labeled; cells clearly identifiable as oligodendrocytes were not labeled, and ependymal cells were rarely labeled.

ER mRNA expressing cells were mapped in the female rat (Fig. 4). Considerable numbers of labeled cells were seen in the periventricular stratum of the POA. They were detected clearly not only in the suprachiasmatic division of the

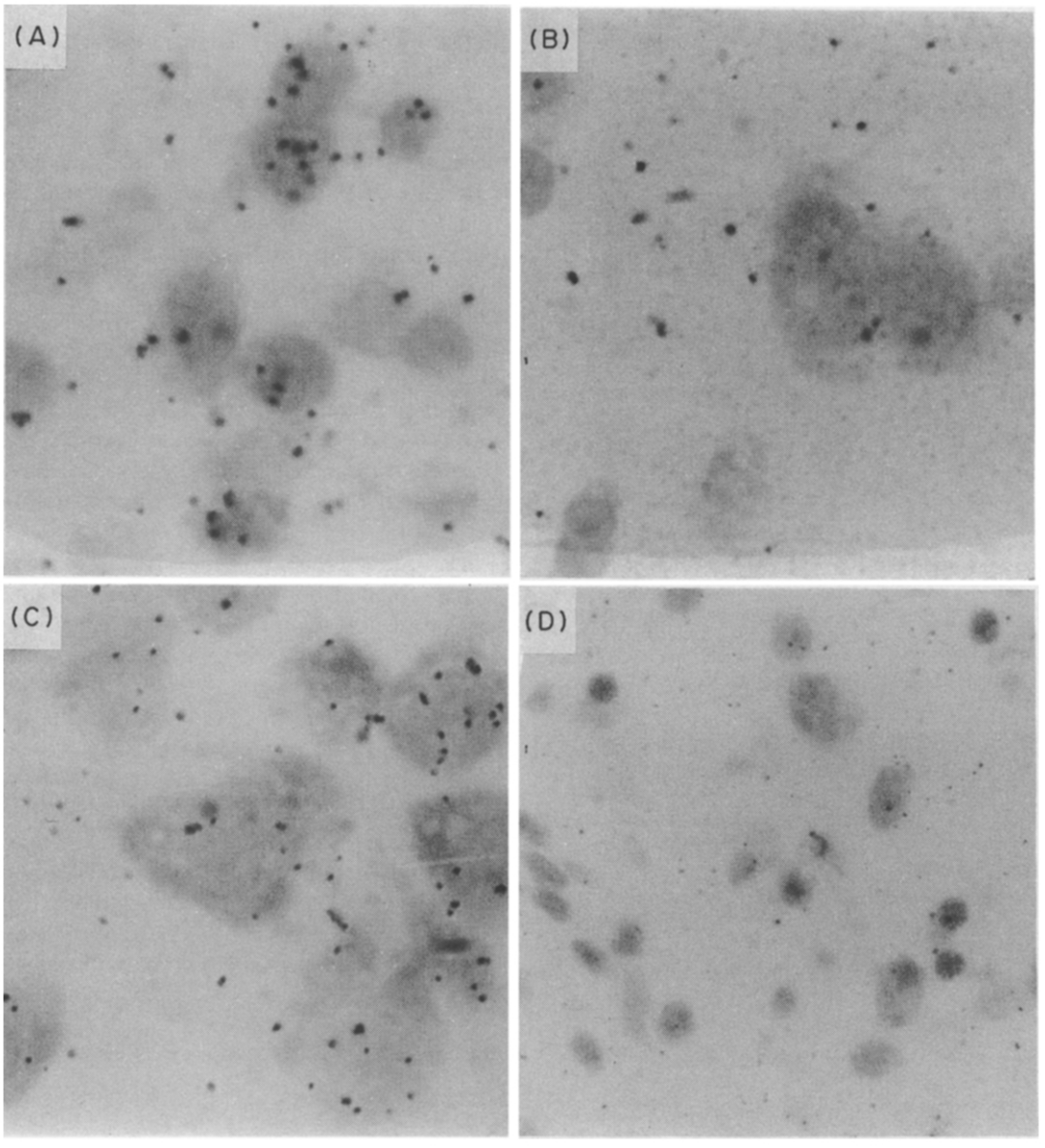

Fig. 3. (A) photomicrograph of cells in the suprachiasmatic portion of the POA. Sections were probed with a [^{3}H]single-stranded DNA synthesized from the cDNA for the human ER, to detect the rare message for ER. There are more grains over cell bodies than over neuropil or cells in the lateral hypothalamus (see methods), demonstrating the capacity of the neurons to express the ER gene. This section is representative of the hybridization for ER mRNA in the POA of ($n = 4$) ovx rats; (B) grains over cells in the cerebral cortex from the same brain section as shown in 3(A), demonstrating the lack of hybridization for ER mRNA in a region which contains relatively few ERs; (C) photomicrograph of cells in the medial preoptic nucleus of an intact male rat. Grains over cell bodies on the right side of the photo show the capacity of these neurons to express the gene for PR in the male, producing message levels which are detectable, but rare compared to those for neuropeptides; and (D) from the same tissue section as 3(C), a control photo showing absence of PR mRNA hybridization amongst cells of the lateral hypothalamic area.

POA, a wedge of cells below the medial preoptic nucleus (MPON), but also in smaller numbers, near the bottom of the brain just lateral to the suprachiasmatic POA (Fig. 4). ER expressing cells were distributed in the MPON in a manner that clearly includes the sexually dimorphic region [32, 46, 47]. While small numbers of labeled cells in the POA could be found outside the MPON, a somewhat larger number was detected in the ventral portions of the bed nucleus of the stria terminalis. Virtually no labeled cells were seen in the lateral preoptic area (LPOA), among the fibers of the medical forebrain bundle.

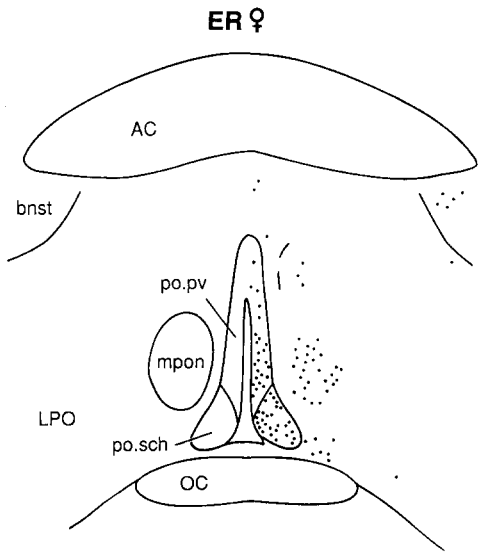

Fig. 4. Chart showing locations of ER mRNA expressing neurons in the POA of ovx female rats. Each dot locates a labeled cell, compiled from 3 sections from 3 different rats. AC, anterior commissure; bnst, bed nucleus of stria terminalis; LPO, lateral preoptic area; OC, optic chiasm; po.pv, preoptic periventricular nucleus; po.sch, preoptic suprachiasmatic nucleus.

PR mRNA expressing cells were distributed very similarly to ER expressing cells in female rats (Fig. 5). Labeled cells were found in the periventricular nucleus (AVPv of Simerly *et al.* [48, 49]). Within the suprachiasmatic portion of the POA, labeled cells tended to be clustered near the dorsomedial tip and near the ventrolateral border. The PR expressing cells were also seen just lateral to this cell group, above the dorsal border of the optic chiasm. Well-labeled cells were detected in the MPON, including, particularly, the medial subdivision. PR expressing cells were not only seen in the ventral portion of the bed nucleus of the stria terminalis, but also in a small group just medial to that, apparently corresponding to the septohypothalamic nucleus (Fig. 5).

In the male POA, the distribution of PR expressing cells was very similar to that of the female (Fig. 6). Notably, all groups of PR expressing cells in the preoptic distribution of the female were also detected in the male. Not only were well-labeled cells seen in the periventricular stratum, but they were also distributed throughout the suprachiasmatic portion of the POA (Fig. 6). With the tritiated PR probe, labeled cells were seen in the MPON, especially near its medial border. PR mRNA containing cells were not always confined within obvious cytoarchitectonic borders, at least as visualized in these thin 10 μ sections. Labeled cells were detected not only in the septohypothalamic nucleus, but also in the ventral portion of the bed nucleus of the stria terminalis (Fig. 6). Quantitatively, the best labeled cells in the intact male MPON (e.g. from 1 animal, mean = 11.8 grains per cell, compared to 0.9 in LPOA) were roughly equivalent to those in the female MPON (e.g. from 1 EB-treated female 7.6 mean grains per cell, compared to 1.1 in LPOA).

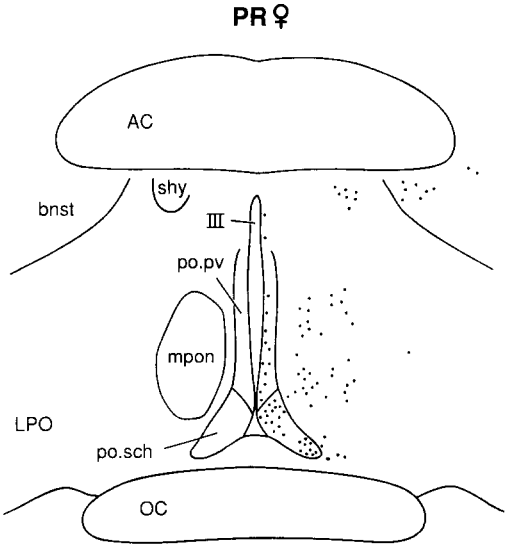

Fig. 5. Chart showing locations of PR mRNA expressing neurons in the POA of estrogen-treated ovx female rats. Each dot locates a labeled cell, compiled from 3 sections from 3 different rats. Shy, septohypothalmic nucleus; for other abbreviations see legend to Fig. 4.

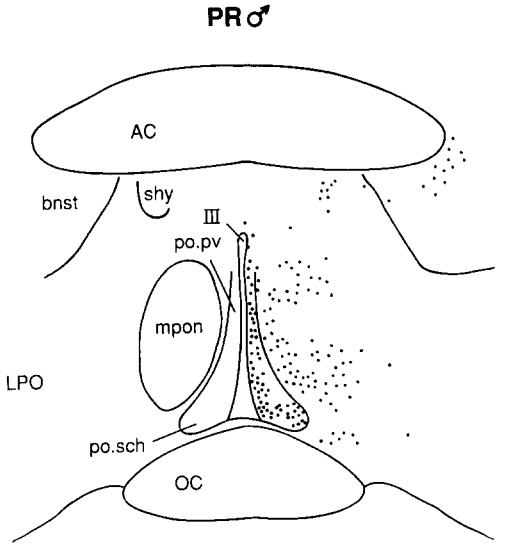

Fig. 6. Chart showing locations of PR mRNA expressing cells in the POA of testosterone-treated males. Each dot locates a labeled cell, compiled for 3 different rats. For abbreviations see legend to Fig. 4.

Throughout the sampling of preoptic tissue, major differences were not detected in the neuroanatomical distributions of ER and PR expressing cells in the female. Likewise, for the PR message, the distribution of labeled cells was very similar between female and male rats.

DISCUSSION

With respect to the preoptic cell groups implicated in the sexually differentiated control of LH release, *in situ* hybridization detected messenger RNAs for both the ER and PR in both neuronal groups; the suprachiasmatic portion of the POA and AVPv, as well as in the sexually dimorphic MPON. In the female rat POA, the distribution of cells expressing ER mRNA was very similar to that of PR mRNA, consistent with receptor autoradiography [37, 50] and immunocytochemical work showing the distribution and colocalization of ER and PR in the POA [51, 52]. Thus, these data show that there is no apparent sex difference in the distribution of cells with the capacity to express the PR gene. Certainly, males do not show a lack of PR mRNA in any particular nucleus where the gene is expressed heavily in the female. However, this analysis may not have been sensitive enough to detect subtle differences in the relative concentration of PR mRNA between males and females within the nuclei of the POA.

With regard to sex differences in the relative concentrations of ER and PR, one study has used autoradiography to assess sex differences in ER concentration; but the data did not address potential differences in cellular distribution [12]. Steroid receptor binding studies have differed in methodology and thus, in the conclusions. For instance, sex differences in ER were not found VMN with larger tissue dissections [10], while different results were found when binding studies were conducted on microdissected tissue [12]. It is interesting to note that, although ER and PR appear to be colocalized within the same neurons [51, 52], males are reported to have fewer ERs than females in the medial POA, while the number of estrogen-induced PR binding sites apparently do not differ [11, 12, 53]. This is not the case, however, in the periventricular preoptic nucleus, where females have been reported to have more ER and PR binding sites than males [10, 12]. Clearly, more research is necessary at the protein and mRNA levels to elucidate the substrate(s) responsible for these differences.

In view of homologies among members of the gene family for steroid receptors, questions of specificity of hybridization must be considered. First, it is highly unlikely that preoptic neurons labeled in the present study actually reflect cross-hybridization for mRNAs for glucocorticoid, mineralacorticoid or thyroid hormone receptor. The probes for the ER and PR messages used here and in previous studies in this laboratory [19–21] have demonstrated anatomically specific patterns which closely match estrogen and progestin binding, while, in contrast, they do not match the widspread distributions of glucocorticoid and thyroid hormone receptor [54, 55]. Secondly, the probes used here and in our previous studies have shown specificity with regard to regulation: estrogen down-regulates the mRNA for its own receptor [19], while it greatly increases the number of neurons in the ventromedial nucleus of the hypothalamus expressing the gene for the PR [20, 21]. Thirdly, the main features of the distributions of cells expressing these messages in the current study match hormone binding results deriving from steroid autoradiography for tritiated estradiol [37] and progestins [50], as well as the distribution of estrogen and progestin receptor containing neurons identified with immunocytochemistry [51, 52, 56]. Thus, the methodology used here is capable of distinguishing neurons expressing messages for different members of the gene superfamily in question.

In situ hybridization as performed in this laboratory is sensitive enough to reveal sex differences in the ER and PR message levels when combined with rigorous quantification. Recently, we have found that the level of ER mRNA of males is 51 and 56% of the amount found in females in the ventromedial and arcuate nuclei, respectively [22]. Further, we have shown that estrogen increases the relative level of PR mRNA in the ventromedial and arcuate nuclei of females while the same hormonal treatment failed to increase the level of PR message in males [21]. Although slot blot hybridization appears sufficiently sensitive for detecting sex differences in higher abundance neuropeptide messages [57], *in situ* hybridization is preferable clearly for quantitating relative levels of the low abundance ER and PR messages.

With respect to the control of ovulation, the current results clearly provide for estrogen- and progesterone-stimulated mechanisms in neurons of the AVPv preoptic cell group [48, 49], the

suprachiasmatic portion of the POA (see for example, Ref. [34]), as well as the MPON itself, a sexually dimorphic structure [58]. In previous physiological work, the problem with distinguishing among these structures has been that all three of these fields of neurons are relatively small and very close in proximity. Thus it is difficult to lesion them with perfect selectivity, and even hormone implant studies are difficult to interpret because of the possible diffusion from one cell group to the next. Nevertheless, no qualitative difference is seen in the present work, for neurons expressing PR and mRNA, between males and females.

Thus, we conclude that the failure of the male rat to generate an ovulatory LH surge is not due to an obvious absence of a subpopulation of PR expressing cells. There are numerous factors which could account for the neuroendocrine deficit. It could be that neurons in the male are less sensitive to neural or hormonal controlling variables within their physiological ranges, or that differences in LH regulation may be the result of neuroanatomical sex differences in the forebrain [59]. Moreover, perhaps another transcription factor cooperating with the PR on its cognate response element, associated with the start site of a regulated gene, could be at lower levels in the genetic male.

The POA appears inhibitory for display of female reproductive behavior in the female rat [60] and to an even greater extent in males [61]. This inhibition is partially overcome in the presence of estrogen, perhaps by decreasing the electrical activity of preoptic neurons [62–64]. However, the relative insensitivity of males to the behavioral effects of progesterone [27, 39, 40] in some cases has been ascribed to differences in estrogen inducibility of progestin receptors in various brain regions [28]. Further studies are needed to clarify the functional importance of, and the mechanisms underlying the apparent sex difference in behavioral responsiveness to estrogen and progesterone (see Ref. [26]).

In summary, the present data show the absence of sex differences in the precise distribution of preoptic cells containing PR mRNA. Rigorous quantitation is required to determine whether or not there are sex differences in the relative numbers of cells expressing the PR gene or in the level of mRNA, and to determine the relationships between the levels of PR and ER mRNA versus the concentration of PR and ER proteins in the forebrain.

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