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Oestrogen Receptor-α and -β Immunoreactivity in Gonadotropin-Releasing Hormone Neurones after Ovariectomy and Chronic Exposure to Oestradiol

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Abstract

Oestradiol exerts negative- and positive-feedback actions on luteinizing hormone (LH) secretion by modulating gonadotropin-releasing hormone (GnRH) release. Furthermore, a chronic increase in circulating oestradiol in either young ovariectomized (OVX) rats, or in middle-aged persistent oestrous (PE) rats, causes a gradual attenuation of LH surges until the positive-feedback action of oestradiol disappears. Based on these findings, and on the equivocal evidence regarding a direct action of oestradiol on GnRH neurones, we tested the hypothesis that chronic oestradiol abolishes LH surges by decreasing the proportion of GnRH neurones containing oestrogen receptor (ER)α or β. Regularly cycling rats were ovariectomized, and half immediately received oestradiol. Three days, or 2 or 4 weeks later, rats were perfused at 18.00 h, and GnRH was colocalized with ERα or ERβ by immunocytochemistry. ERβ was expressed in 76% of GnRH neurones, whereas virtually no GnRH cells were immunopositive for ERα. The proportion of GnRH cells expressing ERα or β in OVX rats was not altered by oestradiol or time after OVX, and this was the case regardless of their medial to lateral, or rostral to caudal location. The results indicate that the mechanisms for the positive-feedback action of oestradiol, and the loss of LH surges in OVX rats after chronic oestradiol, are not mediated by changes in the proportion of oestrogen-receptor containing GnRH neurones.

During the female reproductive cycle, the preovulatory luteinizing hormone (LH) surge, which causes ovulation, is triggered by the positive feedback action of oestradiol on the hypothalamic-pituitary axis (1). The positive-feedback mechanism consists of a preovulatory rise in circulating oestradiol levels (2) that activates the hypothalamic gonadotropin-releasing hormone (GnRH) neurones and causes an increase in GnRH release, which in turn stimulates LH release (3, 4). In rodents, this action of oestradiol is linked to a circadian signal; thus, the LH surge occurs at a specific time of day depending on the photoperiod (5, 6). Throughout the remainder of the oestrous cycle, oestradiol and progesterone exert a tonic inhibition, or negative-feedback action, on the hypothalamic-pituitary axis (7) and suppress LH pulse frequency (8, 9), thereby sustaining basal serum LH concentrations.

With regard to the neuroendocrine control of LH secretion, a major question that remains to be resolved is whether oestradiol exerts part of its positive- or negative-feedback action directly on the GnRH neurones. In support of this possibility, the human GnRH promoter contains functional oestrogen response elements (10). In addition, because oestradiol exerts many of its actions via nuclear receptors, of which two subtypes, oestrogen receptor (ER)α and ERβ, have been identified (11), the expression of oestrogen receptors in GnRH neurones also supports this possibility.

It remains controversial whether the GnRH neurones express one or both subtypes of oestrogen receptor. Although earlier reports indicated that neither ERα mRNA (12, 13) nor ERβ mRNA (12) was expressed by GnRH neurones, more recent findings that GnRH neurones express ERβ mRNA (13, 14) provide strong support for the possibility that oestrogen acts directly on the GnRH neurones. Furthermore, some GnRH neurones accumulate radioactivity in their nuclei after *in vivo* administration of ^{125}I-oestrogen (13), and ERβ immunoreactivity is present in GnRH neurones (15, 16) indicating that the ERβ transcript is translated into functional protein. Although ERα immunoreactivity has been identified in GnRH neurones (17), this finding is difficult to reconcile with the multiple reports of an absence of ERα mRNA in these neurones (12, 13, 18). Thus, the preponderance of evidence available to date suggests that ERβ is the major subtype of oestrogen receptor mediating the actions of oestradiol on GnRH neurones.

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Based on the foregoing considerations, it is likely that oestradiol exerts some of its actions directly on the GnRH neurones. However, it remains to be determined whether the physiological changes in oestradiol levels that induce LH surges alter expression of oestrogen receptors in the GnRH neurones. In this regard, there are no differences in the proportion of GnRH neurones expressing ERβ mRNA after oestradiol treatment of ovariectomized rats (13), or throughout the oestrous cycle in mice (14), although in the latter study ERβ mRNA expression was inversely related to circulating oestradiol levels. By contrast, the effects of oestrogen on the proportion of GnRH neurones expressing immunoreactive ERβ are controversial. Hrabovszky *et al*. (16) reported no difference in the incidence of ERβ expression between untreated and oestradiol-treated ovariectomized rats, whereas Kalló *et al*. (15) found that oestrogen treatment of ovariectomized rats suppresses the number of ERβ-immunopositive GnRH neurones. Therefore, the following study was designed to determine whether the modulation of GnRH neuronal function by oestradiol is mediated in part by modulating the expression of ERs in the GnRH neurones.

A variety of evidence suggests that GnRH neurones are functionally heterogeneous. For example, Fos expression in GnRH neurones during the proestrous LH surge is limited to those near the organum vasculosum of the lamina terminalis (OVLT) and caudal to this structure (19, 20). A limited population of GnRH neurones in the OVLT/preoptic area bind ^{125}I-oestrogen (13). Only the GnRH neurones located in the rostral preoptic area and near the OVLT, most of which are medially located, respond to *N*-methyl-D-aspartate (NMDA) with an increase in GnRH gene expression (21). Similarly, a larger proportion of medial GnRH neurones express the NMDA receptor type 1 (NMDAR1) gene, and have higher basal levels of GnRH mRNA than those located laterally (22). Therefore, we also examined whether the effects of oestradiol on the proportion of GnRH neurones expressing ERs differed among rostral to caudal or medial to lateral subpopulations of GnRH neurones.

We employed ovariectomized rats chronically treated with oestradiol because long-term administration of oestradiol induces daily LH surges that gradually decrease in amplitude and disappear within 2 weeks (6, 23, 24). This loss of response to the positive feedback action of oestradiol is paralleled by a decrease in c-*fos*-activated GnRH neurones (25). Therefore, we tested the hypothesis that, in ovariectomized rats chronically treated with oestradiol, the loss in response to oestradiol, and thereby of the LH surge, is mediated in part by a decrease in the proportion of ERα and/or ERβ-immunopositive GnRH neurones.

Materials and methods

Animals

All procedures were performed according to NIH guidelines and were approved by the University of Kentucky Internal Animal Care and Use Committee. Female rats (aged 60 days) were obtained from Zivic-Miller (Zelienople, PA, USA) and housed under a 14 : 10 h light/dark cycle (lights on at 05.00 h) and controlled temperature (21 °C) with food and water provided *ad libitum*. Vaginal lavages were monitored 6 days per week to determine oestrous cyclicity. All rats in these studies manifested at least three regular 4-day oestrous cycles before use.

Design

To determine whether the loss of LH surges induced by chronic exposure to oestradiol is associated with a decrease in the proportion of ERα-immunopositive GnRH neurones, eight groups of 3–5 regularly cycling Zivic-Miller Sprague-Dawley rats (n = 34) were bilaterally ovariectomized (OVX, day 0). Immediately thereafter, half of the animals (OVX + E_2) received a capsule s.c. containing crystalline 17β-oestradiol (Sigma, St Louis, MO), constructed as described previously with Silastic medical grade tubing (Medical-Tech West, Inc., Hudson, MA, USA) and plugged with silicone elastomer (Factor II, Lakeside, AZ, USA) (26). Such implants have been reported previously to generate sustained plasma oestradiol concentrations averaging approximately 75 pg/ml (26). The remaining half of the animals served as untreated controls. Three days, or 2, 4, or 8 weeks later, all rats received corn oil vehicle (0.1 ml/100 g body weight) s.c. at 12.00 h (because they served as controls for progesterone-treated rats in another study). To determine the effects of exposure to chronic elevations in plasma oestradiol concentrations on LH surges, blood samples (0.2 ml) were obtained hourly from 12.00 h to 19.00 h for characterization of LH surges by means of an atrial cannula that had been inserted on the previous day (27). These results have been reported previously (28). For immunocytochemical localization of ERα and GnRH, the rats were deeply anaesthetized between 17.00 h and 19.00 h and perfused intracardially with Dulbecco's phosphate-buffered saline (0.05 M, pH 7.0, Gibco BRL, Grand Island, NY, USA) followed by 4% paraformaldehyde (0.05 M, pH 7.3–7.4, Fisher, Fair Lawn, NJ, USA) containing 7.5% picric acid (Sigma). The brains were removed, blocked, stored in 25% sucrose at 4 °C, and later cut into 10 series of 40 μm frontal sections on a cryostat. Thus, in a given series, the sections were 400 μm apart. During sectioning, the series containing the first section in which the anterior commissure crossed the midline was identified in each brain. The sections were stored in cryoprotectant (29) at −20 °C. The set of sections 400 μm caudal to the above landmark, which contained the OVLT, was subsequently processed by immunocytochemistry for dual localization of ERα and GnRH using rabbit anti-rat ERα (C1355, Upstate Biochemical, Lake Placid, NY, USA) and rabbit anti-GnRH (LR1, Dr Robert Benoit, Montreal General Hospital, Quebec).

To localize ERβ and GnRH, a rabbit anti-human ERβ antibody (Z8P, Zymed, San Francisco, CA, USA) was employed, which became available after the beginning of the study. Because ERβ could not be detected without acrolein, eight additional groups of 4–6 rats each (n = 16 OVX and n = 21 OVX + E_2) were treated identically, except that they were perfused with 2.5% acrolein (Sigma) in 4% paraformaldehyde to enable detection of ERβ.

To verify that the presence of acrolein in the fixative did not alter the results obtained with ERα, a second set of sections from one rat randomly selected from each group in which ERβ had been localized were processed as above for ERα and GnRH colocalization, except that the anti-ERα was used at a 1 : 30 000 dilution.

Immunocytochemistry

GnRH and ERα or ERβ were localized by means of a previously described dual immunoperoxidase method (19, 30, 31), modified as follows. Brain sections were rinsed in Tris-buffered saline (TBS, 0.05 M, pH = 7.4; Sigma) and then incubated in rabbit antirat ERα (1 : 5000 or 1 : 30 000 C1355, Upstate Biochemical) or ERβ (1 : 1000 Z8P, Zymed) in TBS containing 0.4% Triton X-100 (Sigma) and 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) at 4 °C for 48 h. After thorough rinsing in TBS, the sections were incubated in 1 : 600 goat anti-rabbit biotinylated IgG (Vector) in TBS containing 0.4% Triton X-100 at room temperature for 1 h. After rinsing in TBS, the sections were incubated in 1 : 45 avidin : biotinylated peroxidase solution (Vectastain Elite ABC Kits, Vector) for 1 h at room temperature. The sections were then rinsed in 0.175 M sodium acetate buffer (pH = 6.5). Nickel (0.025 M nickel sulphate)-intensified diaminobenzidine (0.02% DAB, Sigma) was used as the chromogen to yield a blue–black reaction product. After rinsing in TBS to stop the reaction, the tissue sections were incubated in rabbit polyclonal anti-GnRH (1 : 10 000 LR1, a kind gift from Dr Robert Benoit, Montreal General Hospital, Montreal, Quebec) in TBS containing 0.4% Triton X-100 and 10% normal goat serum at 4 °C for 48 h. The procedure for localization of GnRH was essentially the same as that described above, except that DAB was used in order to generate a brown reaction product. Finally, the sections were mounted, air-dried, and coverslipped.

Analysis

Twelve sections from the series beginning 400 μm caudal to the first midline crossing of the anterior commissure for each rat were examined under a light microscope (Eclipse E6000, Nikon, Tokyo, Japan), and all GnRH cells cut through the nucleus were counted at a magnification of ×400 and designated either ERα- or ERβ-positive or -negative. All cell counts were performed without knowledge of the animal's treatment group.

Mean numbers of GnRH neurones, mean numbers of dual-labelled neurones, and mean proportions of dual-labelled neurones were analysed by two-way analysis of variances (ANOVAs) (SigmaStat, SPSS Inc., Chicago, IL, USA) with treatment and

time as the main effects. $P < 0.05$ was considered statistically significant. If a given ANOVA revealed a significant interaction, Tukey's post-hoc tests were performed.

To investigate regional differences in the total number of GnRH neurones, or the proportion of ERβ-immunopositive GnRH neurones in medial to lateral, or rostral to caudal subpopulations, medial GnRH neurones were defined as those within 50 μm of the midline, and included neurones of the medial septum, OVLT and median preoptic nucleus. All remaining GnRH neurones were included in the lateral subpopulation. For the rostral to caudal analysis, the middle region was defined to include four sections: the section containing the OVLT, the section rostral to it, and the two sections caudal to it. All remaining GnRH neurones in a given set were defined as either rostral or caudal to the middle region.

The mean number of GnRH neurones and the mean proportion of dual-labelled neurones between medial and lateral regions were compared by Mann–Whitney Rank Sum tests because the data were not normally distributed. For the same reason, a Kruskal–Wallis ANOVA on Ranks was employed to determine whether the mean number of GnRH neurones differed among rostral, middle and caudal regions. Post-hoc comparisons were peformed using Dunn's method. The mean proportion of dual-labelled neurones was compared from rostral to caudal by a one-way ANOVA with Holm-Sidak post-hoc tests in cases of a significant main effect. Effects of treatment or time on the mean number of GnRH neurones and the mean proportion of ERβ-immunopositive GnRH neurones within each region (medial to lateral and rostral to caudal) were determined by two-way ANOVAs. If an interaction was significant, Tukey's post-hoc tests were performed.

Results

Sections from approximately half of the rats from each group were processed by immunocytochemistry at one time. There was no difference in the number of GnRH neurones between the two runs for either nonacrolein-fixed (51.84 ± 4.44 versus 62.67 ± 4.50 neurones, Run 1 versus Run 2, $P = 0.10$) or acrolein-fixed tissues (91.25 ± 5.46 versus 87.38 ± 4.82 neurones, Run 1 versus Run 2, $P = 0.64$), as determined by t-tests. Therefore, the data from both runs were subsequently analysed together.

Figure 1 depicts the immunocytochemical localization of GnRH and ERα (left panel) or ERβ (right panel) near the OVLT in representative brain sections. An average of 57 neurones was counted in each rat. The total number of GnRH neurones in the sections immunostained for ERα and GnRH does not change with time after ovariectomy or oestradiol treatment (treatment × time, $P = 0.551$) (Fig. 2, upper left panel). Virtually no GnRH neurones express ERα protein (Fig. 2, middle left panel), therefore the proportion of dual-labelled neurones is 0–1% in all groups.

In sections immunostained for ERβ and GnRH, the number of GnRH neurones does not change with time after ovariectomy or oestradiol administration (treatment × time, $P = 0.072$) (Fig. 2, upper right panel). The overall average number of neurones counted was 90 per animal. This number represents the neurones counted in only 10% of the sections, and thus fits with estimates of approximately 1000–1200 GnRH neurones in the intact female rat brain (32). This is greater than that in the nonacrolein fixed sections, and is most likely due to the increased sensitivity attained with acrolein. ERβ is expressed in most GnRH neurones; however, the number of dual-labelled neurones is not altered with time after ovariectomy or oestradiol treatment (treatment × time, $P = 0.443$) (Fig. 2, middle right panel). By contrast to the virtual absence of expression of ERα, the majority (approximately 76%) of GnRH neurones express ERβ, and this proportion is not altered by time after ovariectomy or oestradiol administration (treatment × time, $P = 0.892$) (Fig. 2, lower right panel).

To determine whether the difference in colocalization of ERα and ERβ with GnRH was caused by the use of acrolein during fixation, we determined the proportion of ERα-immunopositive GnRH neurones in an adjacent set of sections from eight of the

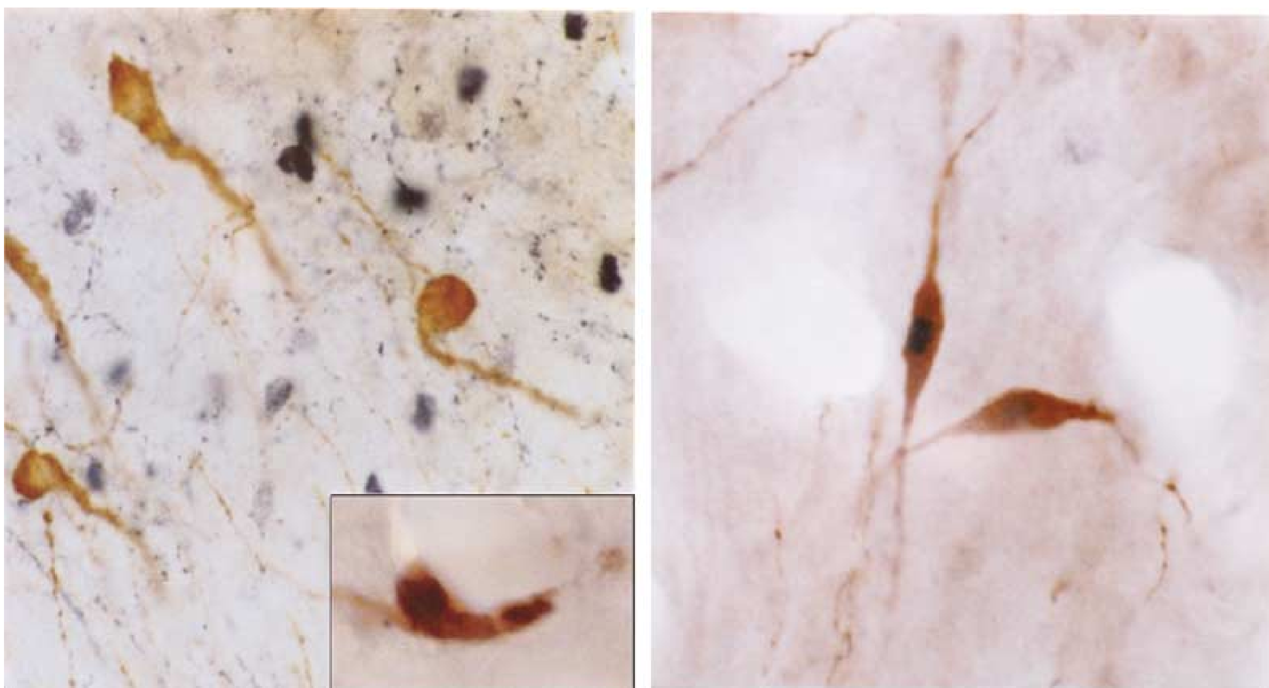

FIG. 1. The left micrograph depicts immunohistochemical colocalization of oestrogen receptor (ER)α (black nuclei) and gonadotropin-releasing hormone (GnRH) (brown cytoplasm) in a representative brain section (×400) containing the organum vasculosum of the lamina terminalis (OVLT). Very few, if any, GnRH neurones express ERα, which is localized in numerous unidentified neurones in the vicinity. An example of a rare ERα-immunopositive GnRH neurone (×1000) is shown in the inset. The right micrograph illustrates colocalization of ERβ (black nuclei) and GnRH (brown cytoplasm) in a representative section (×400) near the OVLT. The majority of GnRH neurones express ERβ.

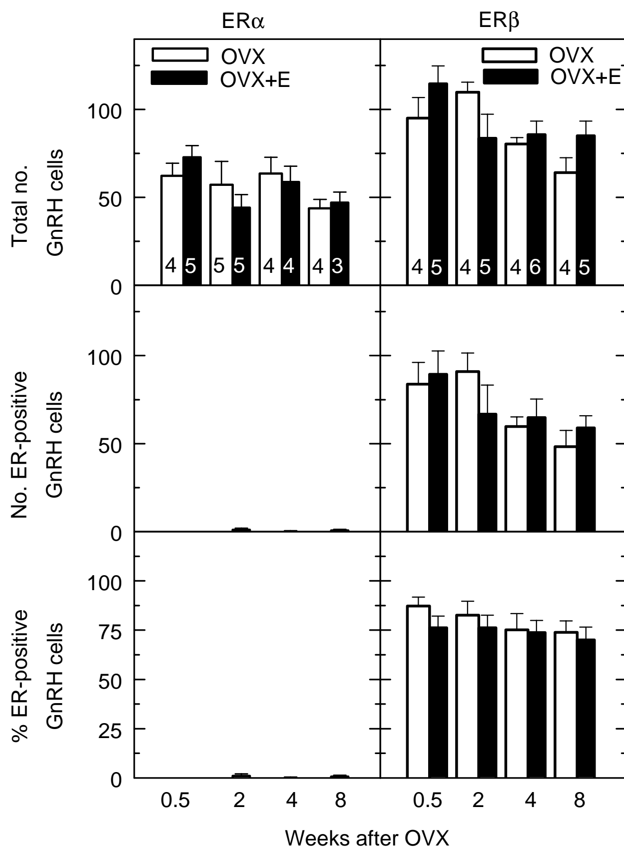

FIG. 2. Lack of an effect of duration of ovariectomy (OVX) or oestradiol treatment (OVX + E) on mean (±SE) total numbers of gonadotropin-releasing hormone (GnRH) immunopositive cells (upper panels), or on mean (±SE) numbers (middle panels) and proportions (lower panels) of oestrogen receptor (ER)α- or ERβ-immunopositive GnRH neurones (left and right panels, respectively). A two-way ANOVA was performed for each panel and there were no significant interactions between treatment and time.

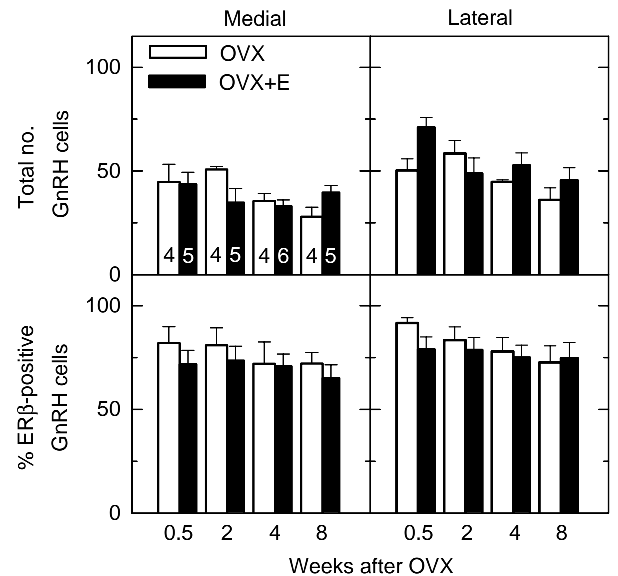

FIG. 3. Absence of a difference between medial or lateral gonadotropin-releasing hormone (GnRH) neurones (left and right panels, respectively) in the effect of ovariectomy (OVX) or oestradiol treatment (OVX + E) on mean (±SE) number of GnRH neurones (upper panels), or on mean (±SE) percent of oestrogen receptor (ER)β-immunopositive GnRH neurones (lower panels). Medial neurones are those within 50 μm from the midline, and include neurones in the medial septum, the median preoptic nucleus, and the region of the organum vasculosum of the lamina terminalis. Two-way ANOVAs were performed on each parameter for each subpopulation and there were no significant interactions between treatment and time.

same rats in which ERβ was localized. In this set of sections, an average of 33 GnRH neurones were counted per rat, in contrast to the average of 90 neurones per rat that were counted in the adjacent set of sections in which ERβ and GnRH were colocalized. This decrease mainly reflects the anatomical distribution of GnRH neurones in the brain because there is a rapid decrease in number of GnRH neurones with distance from the OVLT, and the sections stained for ERα and GnRH were 40 μm caudal to those stained for ERβ and GnRH. Only two GnRH neurones were considered to contain ERα immunoreactivity among 265 GnRH neurones examined, confirming the results obtained in nonacrolein fixed sections, and indicating that the difference in proportions of ERα- versus ERβ-immunopositive GnRH neurones is not due to the use of acrolein during fixation.

In the sections processed for both GnRH and ERβ, there were more lateral than medial GnRH neurones (lateral, 51.35 ± 2.48; medial, 38.49 ± 1.94, mean ± SE; $P < 0.001$), however, there was no effect of time after ovariectomy or duration of oestradiol treatment on the number of medial (treatment × time, $P = 0.086$) or lateral GnRH neurones ($P = 0.113$) (Fig. 3, upper panels). The proportion of ERβ-immunopositive GnRH neurones was not different between the medial and lateral subpopulations of GnRH neurones (lateral, $78.80 \pm 2.22\%$; medial, $73.12 \pm 2.43\%$; $P = 0.13$), and there were no effects of ovariectomy or oestradiol treatment at any time between 3 days and 8 weeks on the proportion of either the medial (treatment × time, $P = 0.928$) or lateral dual-stained neurones ($P = 0.723$) (Fig. 3, lower panels).

When the ERβ and GnRH-immunopositive neurones were analysed from rostral to caudal, the middle subpopulation near the OVLT contained more GnRH neurones than the other two subpopulations (rostral, 18.70 ± 1.53 neurones; middle, 57.70 ± 2.60; caudal, 13.49 ± 1.01; $P < 0.05$). There were no effects of duration of ovariectomy or oestradiol treatment on the number of GnRH neurones in the rostral (treatment × time, $P = 0.351$), middle ($P = 0.061$) or caudal ($P = 0.328$) subpopulations (Fig. 4, upper panels). It is interesting that there was a lower proportion of ERβ-positive GnRH neurones located rostrally ($69.12 \pm 3.33\%$) compared to the middle ($77.60 \pm 2.12\%$, $P = 0.03$) or caudal subpopulations ($83.15 \pm 2.55\%$, $P < 0.001$), which were not different from each other ($P = 0.15$) (Fig. 4, lower panels). However, this difference was not specifically related to oestradiol treatment, because a two-way ANOVA indicated there was no significant interaction between treatments or times in the proportion of rostral ERβ-immunopositive GnRH neurones ($P = 0.428$), as was also the case for the proportions of middle ($P = 0.840$) and caudal ($P = 0.599$) ERβ-positive GnRH neurones (Fig. 4, lower panels).

Discussion

Our finding that the number of GnRH neurones is unaltered between 3 days and 8 weeks after ovariectomy or exposure to oestradiol expands those of previous investigators indicating that the number of GnRH-immunopositive cells is the same in 2-week

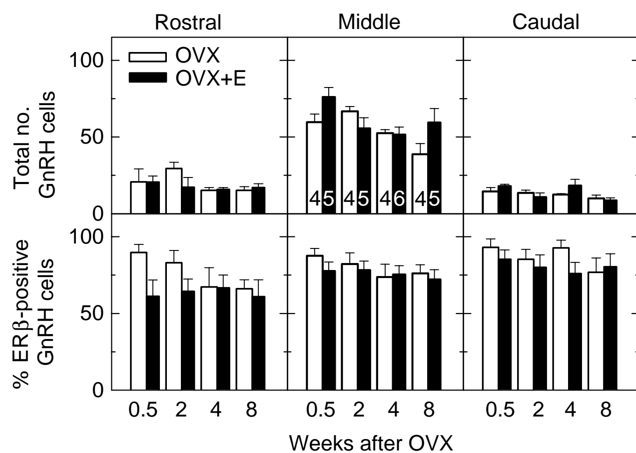

FIG. 4. Lack of a difference among rostral, middle or caudal gonadotropin-releasing hormone (GnRH) neurones (left, middle and right panels, respectively) in the effect of ovariectomy (OVX) or oestradiol treatment (OVX + E) on mean (±SE) number of GnRH neurones (upper panels), or on mean (±SE) percent of oestrogen receptor (ER)β-immunopositive GnRH neurones (lower panels). Middle GnRH neurones were defined as those in the section containing the the region of the organum vasculosum of the lamina terminalis and one section rostral and two sections caudal to that. There were no significant interactions between treatment and time for either parameter in any subpopulation, as determined by two-way ANOVAs.

ovariectomized as in intact proestrous rats (33), and is not altered by 6–7 days of oestradiol treatment in ovariectomized rats (34).

The foregoing results indicate that ERβ is the predominant subtype of oestrogen receptor expressed in GnRH neurones, 76% of which express ERβ. By contrast, virtually no GnRH cells are immunopositive for ERα. These findings confirm previous reports that, in untreated or steroid-treated ovariectomized rats, the majority of GnRH neurones express ERβ (15, 16). With respect to the expression of ERα, although ERα mRNA could not be detected in GnRH neurones from intact (12) or ovariectomized rats by *in situ* hybridization (13), or in GnRH neurones from intact prepubertal mice by reverse transcriptase-polymerase chain reaction (18), immunopositive ERα has been detected in approximately 17% of GnRH neurones from colchicine-treated ovariectomized rats (17). The lack of agreement regarding the expression of ERα may be due to differences in experimental design and/or sensitivity among the methods. However, overall, there is current agreement among recent studies that oestrogen may act directly on GnRH neurones via nuclear receptors and, if so, ERβ may be the predominant subtype mediating oestrogenic control of GnRH neuronal function.

One of the major actions of oestradiol on GnRH neuronal function is to suppress GnRH release by means of a negative feedback action (35, 36), and thereby to maintain basal LH concentrations. At physiologic circulating concentrations of oestradiol, this requires a synergistic action of progesterone; however, at pharmacologic levels, oestradiol alone is sufficient (7). It has yet to be determined whether oestradiol decreases GnRH by a direct action on the GnRH neurones. The results presented herein demonstrate that the proportion of GnRH neurones expressing either ERα or ERβ is not altered up to 8 weeks after ovariectomy. During this period, the plasma LH concentrations in samples obtained from the rats whose brain sections were immunostained for ERα increased approximately five-fold, as reported previously (25). Thus, ERα and ERβ expression remain unchanged even while plasma LH concentrations are increasing. This finding indicates that the postovariectomy increase in GnRH secretion is not mediated by a change in the proportion of GnRH neurones expressing ERα or ERβ.

Another well-established action of oestradiol with respect to GnRH secretion is its positive-feedback action in inducing LH surges, which is mediated by an increase in GnRH release (3, 4, 36, 37). Furthermore, in ovariectomized rats, continuous oestradiol treatment induces a daily LH surge in ovariectomized rats which gradually diminishes until its disappearance approximately 2 weeks after treatment (6, 23, 24). As reported previously (25), a similar gradual attenuation and extinction of oestradiol-induced LH surges occurred in the animals in this study whose brain sections were immunostained for ERα and GnRH. However, the results presented herein indicate that there is no change in the proportion of GnRH neurones expressing ERα or ERβ in response to either a 3-day oestradiol stimulus that induces LH surges, or to a 2–8-week exposure to oestradiol that abolishes LH surges. Therefore, these findings do not support the hypotheses that changes in the proportion of GnRH neurones expressing ERα and/or ERβ play a role in the mechanism whereby acute (3-day) treatment with oestradiol induces LH surges, or in the mechanism whereby chronic exposure to oestradiol (2–8 weeks) abolishes LH surges.

Previous reports disagree as to whether oestradiol administration alters the proportion of ERβ-immunopositive GnRH neurones (15, 16). One possible interpretation, proposed by Kalló *et al.* (15) is that acute treatment with oestradiol decreases the percentage of ERβ-labelled GnRH neurones (i.e. within 3.5 h or 16 h), but chronic exposure to oestradiol, 48 h or longer, has no effect (16) (Fig. 2). Alternatively, the lack of agreement among all the studies may be due to other methodological differences, such as time of perfusion, fixative, duration of ovariectomy, dose of oestradiol, or antibody specificity or sensitivity.

Within the two medio-lateral and three rostro-caudal subpopulations of GnRH neurones, neither the number of GnRH neurones nor the proportion of ERβ-immunopositive GnRH neurones was affected by ovariectomy or oestradiol treatment. However, the proportion of dual-labelled GnRH neurones in the rostral subgroup of GnRH neurones as a whole was lower than in the middle or caudal subgroups. Because this small rostral subpopulation of GnRH neurones is not activated during the LH surge (20), and there were no effects of ovariectomy or oestradiol treatment, this lower expression of ERβ may not be related to the control of LH secretion, but rather to other functions subserved by these rostral neurones that remain to be elucidated.

It is important to note that the methodological approach used in these studies precludes determination of changes in the amount of receptor protein, which may be highly functionally significant in mediating the positive- and negative-feedback effects of oestradiol on GnRH secretion. However, a quantitative analysis of the effect of oestradiol on ERβ protein levels in GnRH neurones has not yet been reported.

In the absence of an oestradiol-induced change in expression of either ERα or ERβ in the GnRH neurones, it remains to be determined whether the positive-feedback actions of oestradiol, or its chronic effect in attenuating and suppressing LH surges in middle age, are exerted directly on the GnRH neurones by postreceptor signalling mechanisms or a change in coregulators in the GnRH neurones, or by nongenomic actions. Alternatively,

the mechanisms for oestrogenic modulation of GnRH, and thereby LH secretion, may be mediated by other neuronal subpopulations that control GnRH secretion. In support of the latter possibility, administration of oestradiol to ovariectomized rats decreases ERα mRNA in the anteroventral periventricular, arcuate and ventromedial nuclei (38–41), and ERα protein in the preoptic area (42).

In conclusion, our results indicate that most GnRH neurones express ERβ. However, neither the postovariectomy increase in GnRH secretion, the positive-feedback action of oestradiol on LH secretion, nor the suppression of LH surges following chronic exposure to oestradiol, is mediated by a change in the proportion of GnRH neurones expressing ERα or ERβ. The functional role(s) of the oestrogen receptors in the GnRH neurones, and the mechanisms whereby the positive- and negative-feedback actions of acute and chronic oestradiol treatment modulate GnRH release in young and ageing animals remain to be determined.

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