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Sex differences in the expression of estrogen receptor alpha within noradrenergic neurons in the sheep brain stem

J.L. Rose [a,b], A.S. Hamlin [a], C.J. Scott [a,b,*]

[a] School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, North South Wales, Australia
[b] Graham Centre for Agricultural Innovation, Charles Sturt University and NSW Department of Primary Industries, Australia

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ABSTRACT

In female sheep, high levels of estrogen exert a positive feedback action on gonadotropin releasing hormone (GnRH) secretion to stimulate a surge in luteinizing hormone (LH) secretion. Part of this action appears to be via brain stem noradrenergic neurons. By contrast, estrogen action in male sheep has a negative feedback action to inhibit GnRH and LH secretion. To investigate whether part of this sex difference is due to differences in estrogen action in the brain stem, we tested the hypothesis that the distribution of estrogen receptor α (ERα) within noradrenergic neurons in the brain stem differs between rams and ewes. To determine the distribution of ERα, we used double-label fluorescence immunohistochemistry for dopamine β-Hydroxylase, as a marker for noradrenergic and adrenergic cells, and ERα. In the ventrolateral medulla (A1 region), most ERα-immunoreactive (-ir) cells were located in the caudal part of the nucleus. Overall, there were more ERα-ir cells in rams than ewes, but the proportion of double-labeled cells was did not differ between sexes. Much greater numbers of ERα–ir cells were found in the nucleus of the solitary tract (A2 region), but <10% were double labeled and there were no sex differences. The majority of ERα-labeled cells in this nucleus was located in the more rostral areas. ERα-labeled cells were found in several rostral brain stem regions but none of these were double labeled and so were not quantified. Because there was no sex difference in the number of ERα-ir cells in the brain stem that were noradrenergic, the sex difference in the action of estrogen on gonadotropin secretion in sheep is unlikely to involve actions on brain stem noradrenergic cells.

1. Introduction

In mammalian females, high levels of circulating estrogen has a positive feedback action in the brain to trigger a massive "surge" release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH), which initiates ovulation [1]. Estrogen also exerts important actions in the brain of males, including the negative feedback regulation of GnRH, and hence LH secretion [2]. The neural basis for this sex difference, whereby estrogen exerts both positive and negative feedback actions on GnRH secretion in females but only a negative feedback action in males remains poorly understood.

In all mammalian species, including sheep, estrogen regulates the secretion of LH from the pituitary gland, in part, through regulation of GnRH secretion from the hypothalamus [1]. Estrogen exerts its actions via the nuclear receptors ERα and ERβ. Studies of knockout mice have confirmed that ERα is critical for the regulation of GnRH secretion, as mice lacking ERβ have normal gonadotropin secretion [3], despite GnRH neurons expressing ERβ [4], but not ERα [5,6]. Thus, it is evident that estrogen must act to regulate GnRH via intermediary system(s) that express ERα.

Neurons that express these estrogen receptors (ER) have been described in various regions within the hypothalamus

* Corresponding author. Tel.: +61 2 6933 4133; fax: +61 2 6933 2587.
E-mail address: chscott@csu.edu.au (C.J. Scott).

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in several mammalian species including sheep, mice, and rats [7–9]. There is also evidence that estrogen can act in the brain stem, where it regulates noradrenergic neurons. Several lines of evidence indicate that these are involved in the regulation of GnRH secretion in female sheep. ERα has been found within noradrenergic neurons in the caudal part of the A1 region of the brain stem [10,11]. The ER-containing noradrenergic neurons in this region project to the medial preoptic area [12,13], the region that contains the GnRH neurons [14] and the bed nucleus of the stria terminalis (BNST) [15], which, in turn, sends projections to the preoptic area [16]. Estrogen treatment of ewes activates noradrenergic neurons [13,15], as indicated by an upregulation of c-Fos within dopamine β-hydroxylase (DBH; a marker for noradrenalin synthesis) immunoreactive (-ir) neurons. In turn, estrogen stimulates the release of noradrenalin within the medial preoptic area, but not in other hypothalamic regions [17]. Furthermore, noradrenergic neurons appear to make contact with the cell bodies and dendrites of GnRH neurons [18] and closely appose them in the median eminence [19].

A functional link between noradrenalin and LH has also been demonstrated, as application of noradrenalin into the medial preoptic area altered LH secretion [20], and central treatment with an α-adrenergic antagonist (doxazosin) blocked the stimulatory actions of estrogen on LH secretion [21]. This body of work collectively demonstrated that, in the ewe, estrogen can act in noradrenalin-producing neurons of the A1 region of the brain stem, which project to the medial preoptic area of the hypothalamus to directly regulate GnRH and hence LH secretion, or indirectly via the BNST. Similarly, there is an increase in noradrenalin release in the median eminence during the preovulatory period in the ewe [22], which correlates with GnRH release. The origin of the noradrenergic projections in the sheep is unknown, but in rats they project from the A1 and A2 regions [23], indicating a second pathway by which estrogen may regulate GnRH secretion via noradrenergic neurons in the caudal brain stem.

In the sheep, there are clear sex differences in the expression of ER messenger RNA (mRNA) in various hypothalamic nuclei [8], including those important for the feedback actions of estrogen, such as the medial preoptic area and the ventromedial nucleus (VMH) [2,24]. Given the evidence for the fundamental role of brain stem noradrenergic neurons in the feedback actions of estrogen in the ewe, we hypothesized that this population of brain stem noradrenergic neurons may also be a key site for this sex difference in estrogen action. To test this hypothesis, we investigated the degree to which ERα is expressed within noradrenergic neurons in the brain stem of rams and ewes.

2. Materials and methods

This study was approved by Charles Sturt University Animal Care and Ethics Committee and was conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Research, outlined by the National Health and Medical Research Council (2004).

2.1. Tissue collection

Brain tissue was obtained from adult merino ewes in the middle of a luteal phase, 10 d after an injection of prostaglandin $F_{2\alpha}$ (Lutalyse; Pfizer, West Ryde, NSW, Australia) and intact merino rams. The stage of cycle was confirmed by visual inspection of the ovaries postmortem. After an intravenous injection of 25,000 IU heparin (Hospira, Mulgrave, Australia), the sheep were killed (sodium pentabarbitone overdose using Lethabarb; Virbac, Peakhurst, Australia) and the heads perfused via the carotid arteries, with 2 L heparinized saline (0.9% sodium chloride with 25,000 IU heparin), followed by 2 L 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 then 1 L of 4% paraformaldehyde and 20% sucrose solution using a peristaltic pump. The brain stem was dissected out to include the proximal 0.5 cm of the spinal cord at the caudal end, through to the corpora quadrigemina at the rostral end. The brain stem was cut into caudal and rostral halves at the caudal end of the cerebral peduncles. The arachnoid mater was removed and brain stem transferred into a solution of PB containing 30% sucrose for 7 d before sectioning in the coronal plane at 40 μm on a cryostat. Sections were stored in a cryoprotectant solution [25] at −20°C. Animals were processed in pairs of a ewe and ram ($n = 6$ per sex). These pairs were maintained for the duration of the study. At tissue collection two of the ewes were found to be pregnant, but the results from these ewes were not different from the remaining ewes.

2.2. Immunohistochemistry

Matched pairs of ewe and ram brain stem sections taken at 720 μm intervals from the spinal cord through to obex and 1,200 μm intervals through the remaining brain stem were mounted on poly-L-lysine (Sigma, St Louis, MO) coated slides. The position of the section on the slide was randomized and coded, to allow for "blinded" cell counting. Sections were given four 10-min washes in 0.05 M phosphate buffered saline (PBS) before mounting, to remove cryoprotectant, and then dried at 37°C overnight. Antigen retrieval was performed using an autoclave (121°C and 15 psi for 30 min in 0.01 M trisodium citrate buffer, pH 6). Sections were then cooled, washed in PBS, and treated for 1 h in blocking solution containing 5% normal horse serum and 0.3% Triton X-100 (Sigma) in 0.1 M PB. The two primary antibodies used in this study were mouse anti-human ERα clone ID5 (Dako, Carpinteria, CA) at 1:100 and rabbit anti-DBH (Protos Biotech, OR) at 1:1000, diluted in 2% normal horse serum, 0.3% triton X-100, 0.1% NaN_3 in 0.1 M PB. Sections were incubated in primary antibody for 60 h at 4°C. Sections were then washed (four, 10 min washes in 0.05 M PBS) followed by incubation in secondary antibodies Alexa 488 goat anti-mouse (Invitrogen, Eugene, OR; 1:200) and Alexa 594 goat anti-rabbit (Invitrogen, Eugene, OR; 1:200), for 1 h. Sections were then washed (four 10-min washes in 0.05 M PBS) before being cover slipped using antifade fluorescent mounting medium (Dako), and stored at 4°C in darkness.

2.3. Quantification of data

Sections were examined using an Olympus BX61 epi-fluorescence microscope equipped with an fView digital