ORIGINAL ARTICLE

Expression of Oestrogen Receptor α in the Brain of Brandt's Voles (*Lasiopodomys brandtii*): Sex Differences and Variations During Ovarian Cycles

Y. Pan*, L. Xu*, Z. Wang† and Z. Zhang*

*State Key Laboratory of Integrated Management of Pest Insects and Rodents in Agriculture, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, Beijing, China.

†Department of Psychology and Program in Neuroscience, Florida State University, Tallahassee, FL, USA.

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Correspondence to:
Z. Zhang, State Key Laboratory of Integrated Management of Pest Insects and Rodents in Agriculture, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, Beijing 100101, China (e-mail: zhangzb@ioz.ac.cn).

Oestrogen receptor (ER) α plays an important role in a variety of cognitive and behavioural functions. It has been shown that ERα expression in the brain is sexually dimorphic and is influenced by circulating oestrogen. In the present study, we mapped ERα-immunoreactive (-ir) cells in the forebrain of Brandt's voles (*Lasiopodomys brandtii*) to examine differences in ERα-ir expression between males and females and to reveal variations of ERα-ir expression during ovarian cycles in females. ERα-ir cells were found in many forebrain regions, including the lateral septum, bed nucleus of the stria terminalis, medial preoptic area (MPOA), anterior, arcuate and ventral medial (VMH) nuclei of the hypothalamus, as well as medial (MeA) and anterior cortical nuclei of the amygdala. Females had more ERα-ir cells in the VMH than males. Females during ovarian oestrus, but not di-oestrus or pro-oestrus, also had more ERα-ir cells in the MPOA than males. Furthermore, females in ovarian di-oestrus or oestrus had more ERα-ir cells in the MeA than males. Together, these data indicate that ERα expression in the brain of Brandt's voles is sexually dimorphic in specific brain areas. In addition, variations in the levels of circulating oestrogen during ovarian cycles can affect ERα expression in the female brain in a region-specific manner.

Key words: oestrogen receptor α, sex difference, ovarian cycle, amygdala, medial preoptic area, ventromedial hypothalamus.

The term 'oestrogen receptor' (ER) refers to a group of receptors that are activated by oestrogen (1). These receptors are classified into the nuclear receptors, ERα and ERβ, which interact directly with oestrogen-regulated genes, and a membrane-associated receptor, which appears to mediate nongenomic effects (2,3). Among the three subtypes of the receptors, ERα is the most studied and has been found in various brain regions, including the hippocampus, hypothalamus and cortex in a variety of animal species examined (4–6). The distribution pattern of ERα has been found to be sexually dimorphic in some brain regions. For example, female rats have more cells labelled for ERα mRNA and ERα immunoreactivity in the medial preoptic area (MPOA) than males (7,8). In addition, regional ERα density is related to the levels of circulating oestrogen: ERα fluctuates throughout ovarian cycles (8,9) and ERα levels increase by ovariectomy (9,10) and decrease following a subsequent oestrogen treatment (9).

ERα has been implicated in a variety of physiological and behavioural functions both during development and in adulthood. For example, neonatal blockade of ERs using ER antagonists or ER antisense oligodeoxynucleotides impaired growth and differentiation of the sexually dimorphic nucleus in the preoptic area and had permanent effects on oestrogen-mediated behavioural differentiation in adulthood in rats (11,12). ER knockout mice showed deficits in their mating behaviour (13,14), aggression (14,15) and social recognition (16), compared to wild-type controls. Behavioural studies have also demonstrated the role of ERs in social affiliation, aggression, infanticide and maternal behaviour in several rodent species (14,17–20). Furthermore, ERα may regulate social behaviours via actions on other neurotransmitter systems (21,22). For example, expression of the neuropeptide arginine vasopressin (AVP) in certain brain areas is steroid-dependent (23–25). ERα levels affect AVP
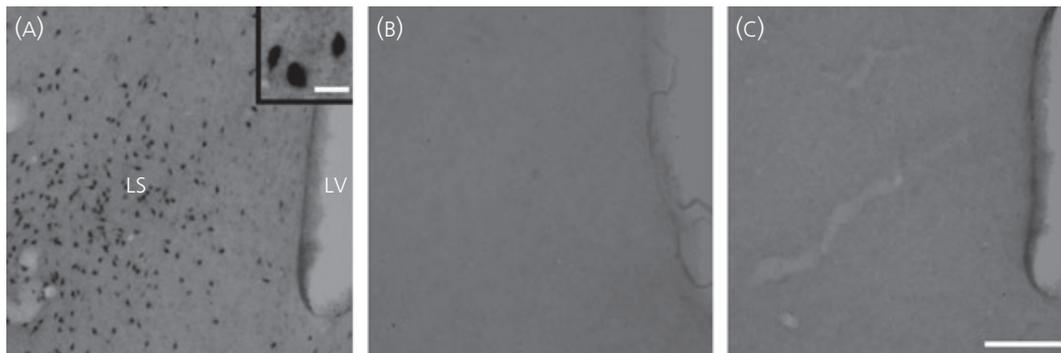
Fig. 1. Photomicrographs displaying oestrogen receptor (ER) α-immunoreactive specific staining in the lateral septum (LS) in the brain of Brandt's voles. ERα immunocytochemistry resulted in dense nuclear staining for cells in the LS (A). The omission of the primary antibody (B) or pre-adsorption of the brain tissue with the 10 × concentrated synthetic peptide against which the antibody was raised (C) led to no specific staining on the brain sections. LV, lateral ventricle. (A–C) Scale bar = 100 μm. (A, inset) Scale bar = 10 μm.

expression (26,27) which, in turn, alters AVP-mediated behaviours such as aggression and affiliation (28,29).

Brandt's voles (*Lasiopodomys brandtii*) are distributed in typical steppes in Inner Mongolia of China, Mongolia, and the region of Beigaer in Russia (30,31). They are social animals that live in large family groups and display extensive social interactions among individuals (32–34). Morphological and behavioural sex differences are found in Brandt's voles, with males being larger and displaying higher levels of territorial defense than females (32,33,35). In a recent study, we found a species-specific pattern of AVP and oxytocin immunoreactive staining in the brain of Brandt's voles, which may play an important role in the regulation of their social behaviours (36). However, despite a higher density of oxytocin immunoreactive (-ir) cells in the paraventricular nucleus of the hypothalamus (PVN) in females than in males, no other sex differences were found—suggesting that sex differences in central AVP and oxytocin systems may not fully explain sex differences in social behaviours. Because central ERα has been implicated in sex-specific reproductive function and social behaviour in other rodent species (13,14,37,38), in the present study, we tested the hypothesis that male and female Brandt's voles differ in the distribution pattern of ERα in the brain. In addition, because circulating oestrogen affects ERα expression (8,39,40) and oestrogen levels fluctuate during ovarian cycles (8,41), we also tested the hypothesis that regional ERα expression in the brain changes during ovarian cycles in female voles, which, in turn, may contribute to sex differences in region-specific ERα expression.

Materials and methods

Subjects

Subjects were adult male and female Brandt's voles (*L. brandtii*) that were offspring of a laboratory breeding colony. The colony was started with field captured animals and maintained in the Institute of Zoology at the Chinese Academy of Sciences in Beijing, China. Subjects were housed in same-sex groups, consisting of two to three individuals each, in plastic cages (25 × 14 × 14 cm). The cages contained wood shavings as bedding and

were maintained under a 16 : 8 h light/dark cycle (lights on 05.00 h). Food and water were provided *ad lib*. Room temperature was maintained at approximately 20 ± 2 °C. All experimental procedures for animal use and care complied with the regulations by the Institute of Zoology at the Chinese Academy of Sciences.

Tissue preparation

Subjects were deeply anaesthetised with sodium pentobarbital (3 mg/100 g body weight, Sigma-Aldrich, St Louis, MO, USA) and perfused through the ascending aorta with 0.1 M phosphate buffered solution (PBS; pH = 7.2) followed by 4% paraformaldehyde in PBS. Brains were quickly removed, post-fixed in 4% paraformaldehyde for 12 h, and then stored in 30% sucrose in PBS. Coronal brain sections, 40 μm thick, were cut on a cryostat from rostral to caudal to the amygdala. Brain sections at 240-μm intervals were processed for ERα immunocytochemistry.

ERα immunocytochemistry

Floating brain sections were processed for ERα immunocytochemistry using an established method (42). Briefly, brain sections were pre-treated with

Table 1. Number of Oestrogen Receptor α-Immunoreactive Cells in the Brain of Brandt's Voles

Brain area	Male	Female	t-test
LS	183.1 ± 11.8	190.5 ± 17.2	NS
BST	748.1 ± 107.6	851.1 ± 37.7	NS
MPOA	505.8 ± 36.1	573.3 ± 20.6	NS
AH	42.5 ± 6.1	37.6 ± 3.1	NS
VMH	310.5 ± 11.6	374.0 ± 30.6	$P < 0.05$
ARC	488.9 ± 12.2	449.8 ± 26.9	NS
MeA	285.1 ± 51.4	361.2 ± 28.8	NS
CoA	245.1 ± 29.3	226.2 ± 27.8	NS

LS, lateral septum; BST, bed nucleus of the stria teminalis; MPOA, medial preoptic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus of the hypothalamus; MeA, medial nuclei of the amygdala; ACo, anterior cortical nuclei of the amygdala. NS, not significant.

Di-oestrus	Pro-oestrus	Oestrus	Male
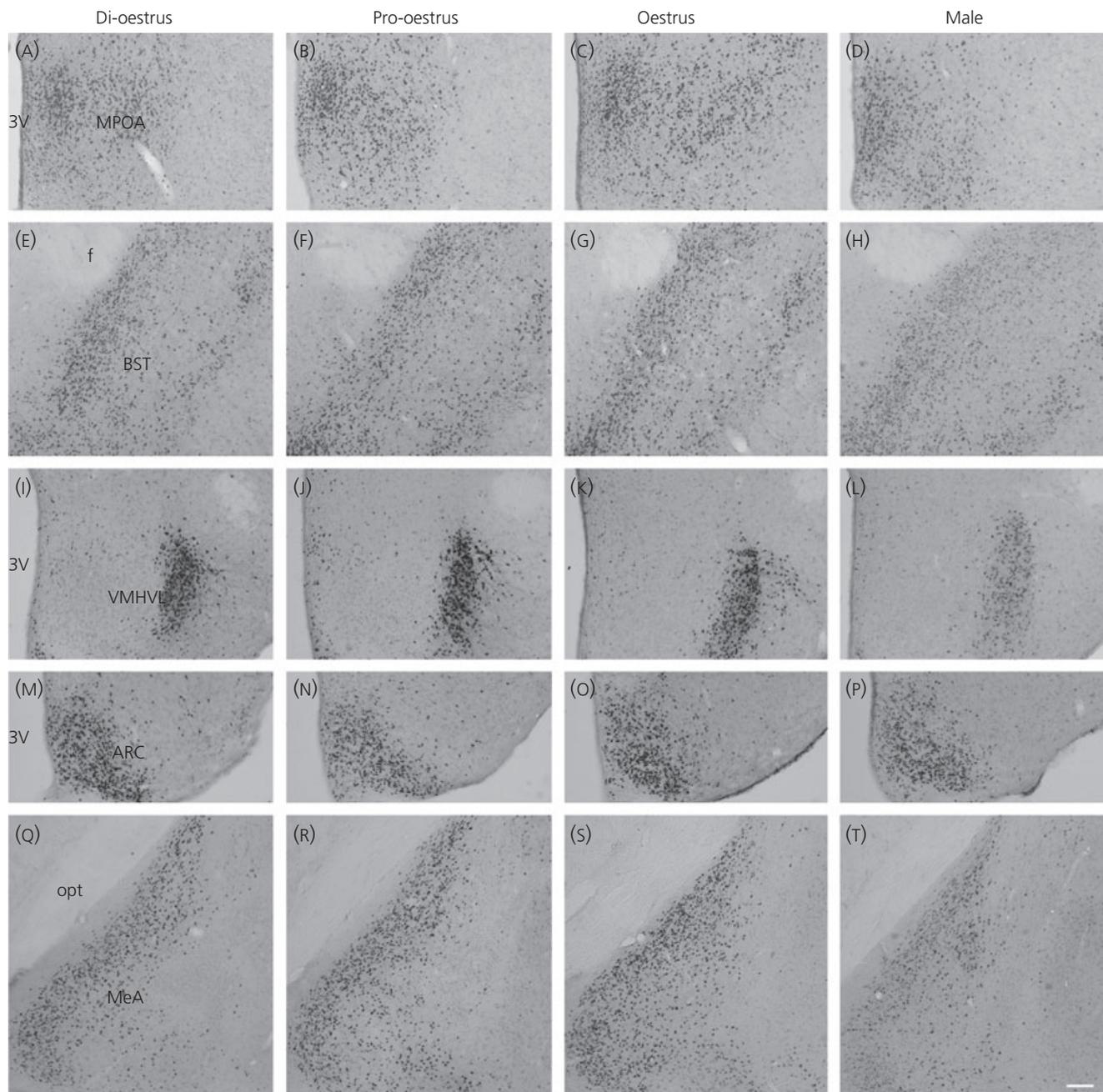
Fig. 2. Photomicrographs displaying oestrogen receptor (ER) α-immunoreactive cells in the medial preoptic area (MPOA; A-D), bed nucleus of the stria terminalis (BST; E-H), ventral medial hypothalamus – ventral lateral part (VMHVL; I-L), arcuate nucleus (ARC; M-P) and medial nucleus of the amygdala (MeA; Q-T) in the brain of Brandt's voles. Subjects were females in dioestrus (A, E, I, M, Q), pro-oestrus (B, F, J, N, R) or oestrus (C, G, K, O, S) of the ovarian cycle and males (D, H, L, P, T). f, fonix; opt, optic tract; 3V, third ventricle. Scale bar = 100 μm.

10 mM citrate buffer for 10 min at 90 °C, followed by 0.5% NaBH₄ for 5 min, and then 0.5% H₂O₂ in 0.1 M PBS for 30 min. Thereafter, sections were treated with PBS with 0.6% Triton X-100 (PBT) for 20 min and then blocked in 10% normal goat serum (NGS) in PBT for 30 min and incubated in rabbit ERα polyclonal antibody (dilution 1 : 8000, C1355, Upstate, Millipore, Billerica, MA, USA) in PBT with 2% NGS for 36 h at 4 °C and an additional 1 h at room temperature. Sections were then incubated with biotinylated goat-anti rabbit secondary antibody (dilution 1 : 300, Vector Laboratories, Inc. Burlingtone, CA, USA) in PBT for 2 h, ABC complex (Vector Laboratories) in PBS for 90 min, and stained by nickel-3,3′-diaminobenzidine. Sections were mounted, air-dried, and coverslipped. This antibody was generated against the last 15 C-terminal amino acids of the rat ERα protein, comprising a region that shares no homology with ERβ. The specificity of this antibody was tested by omission of the primary antibody and by pre-adsorption with the 10 × concentrated synthetic peptide against which the antibody was raised. No specific staining was observed in either case (Fig. 1). To reduce variability in the staining, brain sections within each experiment were processed concurrently.

Data quantification and analysis

All slides were coded to conceal group identity. Slides were inspected under a Nikon microscope (Nikon, Tokyo, Japan) to identify forebrain regions with ERα-ir staining. ERα-ir cells were counted in the lateral septum (LS), bed nucleus of the stria teminalis (BST), MPOA, anterior hypothalamus (AH), ventromedial hypothalamus (VMH), arcuate nucleus of the hypothalamus (ARC), and medial (MeA) and anterior cortical (ACo) nuclei of the amygdala. Brain sections were matched between animals and two or three sections per brain area were examined. ERα-ir cells within each brain area were quantified bilaterally. Group differences in the number of ERα-ir cells in each brain area were analysed by either a t-test or one-way ANOVA followed by a Duncan post-hoc test. P < 0.05 was considered statistically significant.

Experiment 1: Do males and females differ in ERα expression in the brain?

Because sexual dimorphisms were found in ERα labelling in the brain in several rodent species (5,7,8), we hypothesised that male and female Brandt's voles may differ in ERα expression in the brain. To test this hypothesis, sexually naive male (n = 8) and female (n = 8) voles were anaesthetised and perfused, their brains were harvested, and brain sections were processed for ERα immunocytochemistry. The number of ERα-ir cells was quantified in selected brain areas, and sex differences were analysed by a t-test.

Experiment 2: Does ERα expression change during ovarian cycles in females?

Levels of circulating oestrogen change during ovarian cycles in rodents (8,41). Such changes in oestrogen levels have been associated with altered ERα expression in the brain (8,43). In addition, ovariectomy and oestrogen treatment were found to influence ERα expression in selected brain areas (8,39,40,44). Accordingly, we hypothesised that the ERα labelling in the brain of female Brandt's voles changes during ovarian cycles, which, in turn, may contribute to sex differences in ERα expression in the brain. To test this hypothesis, we monitored a group of female voles to determine the stages of their ovarian cycles by performing vaginal cytology. Vaginal di-oestrus was characterised by the presence of leucocytes with few epithelial cells in vaginal smears; pro-oestrus was indicated by mainly epithelial cells with marked nuclei; and oestrus was determined by predominantly cornified epithelial cells (45). Each female was monitored for two cycles to ensure reliable assessment of vaginal stages. Females in vaginal di-oestrus (n = 6), pro-oestrus (n = 7) or oestrus (n = 7), together with a group of males (n = 8), were anaesthetised and perfused. Their brain sections were processed for ERα immunocytochemistry. Group differences in the number of ERα-ir cells in selected brain areas were analysed by one-way ANOVA followed by a Duncan post-hoc test.

Results

ERα-ir labelling in the brain of male and female voles

ERα immunocytochemistry resulted in the specific nuclear staining of cells (Fig. 1A) in many forebrain areas in Brandt's voles. Dense clusters of ERα-ir cells were found in the LS, BST, MPOA, AH, VMH, ARC, MeA and CoA of the amygdala in both male and female voles. Sex differences in the number of ERα-ir cells were analysed for the above mentioned brain areas (Table 1). In the VMH, females had more ERα-ir cells than males. The similar trend was also found in the BST, MPOA and MeA, although such differences did not reach statistical significance.
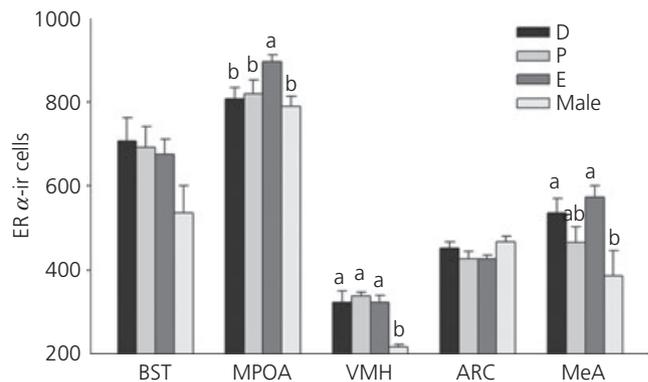
Fig. 3. Group differences in the number of oestrogen receptor (ER) α-immunoreactive (-ir) cells in select forebrain areas in male and female Brandt's voles. In the medial preoptic area (MPOA), females in ovarian oestrus (E) had more ERα-ir cells than males and females in di-oestrus (D) or pro-oestrus (P). In the ventral medial hypothalamus (VMH), females had more ERα-ir cells than males. In the medial nucleus of the amygdala (MeA), females in di-oestrus and oestrus had more ERα-ir cells than males. No group differences were found in the bed nucleus of the stria terminalis (BST) or arcuate nucleus of the hypothalamus (ARC). Alphabetic letters indicate the result from the Duncan post-hoc test following one-way ANOVA. Bars with different letters are significantly different from each other.

Variations in ERα-ir labelling during ovarian cycles in female voles

The majority of female Brandt's voles showed 4- or 5-day ovarian cycles and the average was 4.19 ± 0.23 days. Variations in the number of ERα-ir cells were found during ovarian cycles in female voles in a brain region-specific manner. In the MPOA, more ERα-ir cells were present in samples taken from females in ovarian oestrus than those taken from females in di-oestrus or pro-oestrus or from males ($F_{3,24} = 3.31$, P < 0.05) (Figs 2A-D and 3). In the MeA, females in di-oestrus or oestrus, but not pro-oestrus, had more ERα-ir cells than did males ($F_{3,24} = 3.61$, P < 0.05) (Figs 2Q-T and 3). In the VMH, the number of ERα-ir cells did not differ among females at different stages of ovarian cycles but showed a robust sex difference: females had more ERα-ir cells than males ($F_{3,24} = 14.56$, P < 0.01) (Figs 2I-L and 3). A similar pattern was also found in the BST, although the group difference did not reach statistical significance ($F_{3,24} = 2.34$, P < 0.10) (Figs 2E-H and 3). In the ARC, the number of ERα-ir cells showed no group differences (Figs 2M-P and 3).

Discussion

Brandt's voles display high levels of social interactions with conspecifics, and some of those social behaviours are sexually dimorphic (32, 33, 35, 46). In the present study, we examined the brain expression of ERα, comprising an oestrogen receptor that has been implicated in the regulation of a variety of social behaviours (13,14,37,38). ERα immunocytochemistry produced dense nuclear staining for cells in many forebrain regions, including the LS, BST, MPOA, ARC, VMH,

MeA and CoA, in both male and female Brandt's voles. This distribution pattern of ERα is similar to the pattern reported in other rodent species (4, 6, 47, 48). In addition, our data indicate that regional expression of ERα-ir cells changed during ovarian cycles in female voles, and male and female voles showed a sexual dimorphism in ERα expression in a brain region-specific manner.

A clear sexually dimorphic pattern of ERα expression was found in the VMH in Brandt's voles: females had more ERα-ir cells than males. Interestingly, this sex difference persisted regardless of female's ovarian status. These data suggest that changes in circulating oestrogen during ovarian cycles had no effects on the expression of ERα-ir staining in the VMH in female voles. Thus, this sex difference in ERα expression is most likely determined genetically or by endogenous factors such as gonadal steroid hormones during early development (i.e. the organisational effect of hormones). This notion is supported by data from previous studies in other rodent species. In rats, mice and guinea pigs, the amount of ERα mRNA or protein in the VMH was found to be higher in females than in males (49–52). In another study, the number of ERα-ir cells in the VMH was significantly higher in female rats than in males during early development (7). Further, oestrogen treatment decreased ERα mRNA expression in the VMH in young female rats but was ineffective in middle- and old-aged females (53), suggesting that sex differences in the VMH may be determined during early development. The VMH is critical in the control of male and female reproductive behaviour (54,55) and, therefore, the sexually dimorphic expression of ERα in the VMH may modulate the effects of oestrogen on reproductive behaviour differently between males and females (56).

Although there was approximately 27% more ERα-ir cells in the MeA in female voles than in males in Experiment 1, this difference was not statistically significant. However, when ovarian status in females was precisely monitored, a sexual dimorphism emerged: females during ovarian di-oestrus and oestrus, but not pro-oestrus, had more ERα-ir cells in the MeA than males. It is known that oestrogen levels are higher during ovarian pro-oestrus compared to di-oestrus and oestrus in many female rodents (8,41), and thus it is possible that elevated oestrogen during pro-oestrus induced a decrement in the receptor protein and thus diminished sex differences in ERα-ir labelling in the MeA. This notion is supported by data from previous studies in female rats and guinea pigs showing down-regulation of ERα in the MeA after oestrogen administration (57,58).

Similarly, ERα-ir labelling in the MPOA did not differ significantly between males and females in our Experiment 1. In Experiment 2, however, females in ovarian oestrus had more ERα-ir cells in the MPOA than males. During ovarian oestrus, circulating levels of oestrogen decrease to a level lower than that of pro-oestrus (45). Because oestrogen has been reported to down-regulate ER mRNA or ER protein in the MPOA (39,58), the increase in ERα-ir staining in the MPOA during female ovarian oestrus may result from attenuation of oestrogen down-regulation of ERα expression during pro-oestrus. A similar finding was found in an early study in rats in which ERα mRNA levels were low in pro-oestrous females and males, and high in oestrous females (8). In the same study, however, the level of ERα mRNA in the MPOA of di-oestrous females

was similar to that of oestrous females, both of which were higher than that of males (8). There is no ready explanation for this discrepancy between the current and previous studies; however, species differences may play a role. In addition, ER protein levels do not necessarily correlate with ER mRNA expression (59,60), and thus, differential transcriptional versus post-transcriptional processes may also contribute to this discrepancy.

One caveat in the present study is that the levels of circulating oestrogen during ovarian cycles were not measured from our subjects. No related information can be found from previous studies. Therefore, although the vaginal cytology and the length of ovarian cycles in Brandt's voles are similar as in rats, we still cannot exclude the possibility that the levels of circulating oestrogen during ovarian cycles in Brandt's voles differ from that of rats, which needs to be examined in further studies.

In summary, the data reported in the present study illustrate the distribution pattern of ERα-ir cells in the forebrain of Brandt's voles and reveal differences in the ERα-ir expression in selected brain regions between male and female voles. Furthermore, because regional ERα-ir changes during ovarian cycles in female voles, the sexually dimorphic pattern of ERα expression depends upon a female's ovarian status and is brain region-specific. These data further indicate the importance of monitoring a female's physiological status for studies focusing on male and female comparisons. Finally, ERα plays an important role in a variety of cognitive and behavioural functions (27,61), whereas the VMH, MPOA and MeA have been implicated in social behaviours, including reproductive, affiliative and aggressive behaviours (5,55,62,63). Therefore, further studies should examine the functional (i.e. behavioural) significance of the sexually dimorphic ERα expression between male and female voles.

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