Activation of cells containing estrogen receptor alpha or somatostatin in the medial preoptic area, arcuate nucleus and ventromedial nucleus of intact ewes during the follicular phase, and alteration after lipopolysaccharide.

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ABSTRACT

Cells in the preoptic area (POA), arcuate nucleus (ARC) and ventromedial nucleus (VMN) that possess estrogen receptor α (ERα) mediate estradiol feedback to regulate endocrine and behavioral events during the estrous cycle. A percentage of ERα cells located in the ARC and VMN express somatostatin (SST) and are activated in response to estradiol. The aims of the present study were to a) investigate the location of c-Fos, a marker for activation, in cells containing ERα or SST at various times during the follicular phase, and b) determine if lipopolysaccharide (LPS) administration, which leads to disruption of the LH surge, is accompanied by altered ERα and/or SST activation patterns. Follicular phases were synchronized with progesterone vaginal pessaries and control animals were killed at 0, 16, 31 or 40 h (n=4-6/group) after progesterone withdrawal (PW; time zero). At 28 h, other animals received LPS (100 ng/kg) and were subsequently killed at 31 h or 40 h (n=5/group). Hypothalamic sections were immunostained for c-Fos and ERα or SST. LH surges occurred only in control ewes with onset at 36.7±1.3 h after PW: these animals had a marked increase in the percentage of ERα cells that co-localized c-Fos (%ERα/c-Fos) in the ARC and mPOA from 31 h after PW and throughout the LH surge. In the VMN, %ERα/c-Fos was higher in animals that expressed sexual behavior compared to those that did not. SST cell activation in the ARC and VMN was greater during the LH surge compared to other stages in the follicular phase. At 31 or 40 h after PW (i.e., 3 or 12 h after treatment, respectively), LPS decreased %ERα/c-Fos in the ARC and the mPOA but there was no change in the VMN compared to controls. The %SST/c-Fos increased in the VMN at 31 h after PW (i.e., 3 h after LPS) with no change in the ARC compared to controls. These results indicate that there is a distinct temporal pattern of ERα cell activation in the hypothalamus during the follicular phase, which begins in the ARC and mPOA at least 6-7 h before the LH surge onset, and extends to the VMN after the onset of sexual behavior and the LH surge. Furthermore, during the surge, some of these ERα activated cells may be SST secreting cells. This pattern is markedly altered by acute LPS administered during the late follicular phase indicating that the disruptive effects of this stressor are mediated by suppressing ERα cell activation at the level of the mPOA and ARC, and enhancing SST-cell activation in the VMN, leading to the attenuation of the LH surge.

INTRODUCTION

The ovarian steroid hormone estradiol is of central importance in the control of the hypothalamic-pituitary-gonadal (HPG) axis in female mammals. For the greater part of the ovarian cycle in ewes, progesterone and estradiol act synergistically to restrain GnRH/LH (gonadotropin releasing hormone/luteinizing hormone) secretion through a negative feedback. However, during the late follicular phase, minute-by-minute portal blood sampling in conscious ewes revealed a 'switch' from inhibition to enhancement of GnRH secretion [1, 2]. This constitutes estradiol positive feedback and triggers the onsets of GnRH/LH surge secretion. Steroid hormone signals do not impinge directly on GnRH cells as these cells do not possess progesterone receptors (PR) or estrogen alpha receptors (ERα) [3-5]. Some GnRH neurons express ERβ [6], although it is unlikely that ERβ plays a major role in the feedback regulation of GnRH/LH secretion because ERβ knock-out mice have normal fertility [7, 8].

The surge generating mechanism has been well characterized in the ovariectomized (OVX) ewe [1] and consists of three phases: i) activation phase, during which estradiol concentrations reach a threshold and must remain elevated for a few hours [9, 10]. This signal is 'perceived' by neuronal cells that contain ERα and respond by becoming activated; ii) transmission phase, during which the activation signal is transmitted from ERα cells to GnRH neurons, either directly or *via* one or more interneurons; and iii) surge secretion phase, during which there is a discharge of GnRH and LH [1]. The decrease in plasma progesterone concentrations after luteolysis and the increase in estradiol are also responsible for changes in sexual behavior [11-13].

To date, studies using localized implants have demonstrated that estradiol acts in the mediobasal hypothalamus (MBH; vicinity of the VMN/ARC) to induce both the surge and sexual behaviors [14, 15]. However, we still don't know the precise location and timing of cell activation within the areas involved at each stage of the surge- and behavior-generating mechanisms in response to changes in the steroid hormone milieu. These areas contain several types of neurons, sub-populations of which co-

localize ERα and/or somatostatin (SST)[16]. In the ARC of the sheep, 13% of the SST neurons express ERα [17], and in the VMN, 30% SST neurons express ERα, and this accounts for 70% of the total number of ERα cells in this area [17, 18]. Furthermore, studies carried out in OVX ewes reveal an increase in SST activation after estradiol treatment [17, 19, 20]. Therefore, SST cells are potential candidates as intermediaries between ERα in the control of GnRH secretion and/or sexual behavior.

There is considerable evidence that various types of stressors can disrupt the follicular phase of the ovarian cycle and block or delay the LH surge [21]. For instance, we have recently shown that a sudden activation of the hypothalamus-pituitary-adrenal axis in the late follicular phase by the immunological stressor endotoxin (i.e., lipopolysaccharide; LPS) lowered plasma estradiol concentrations and delayed the onsets of pre-copulatory behaviors, estrus and the LH surge in intact ewes [22]. Furthermore, immunohistochemical analysis of c-Fos protein expression (a marker of neuronal activation; [23]) revealed that this disruption entailed activation of unknown cell types located in the ARC, mPOA and VMN [24]. In considering potential pathways by which stressors disrupt the follicular phase and sexual behavior, four distinct mechanisms may be involved: i) suppression of steroidogenesis at ovarian level; ii) suppression of GnRH pulsatility (frequency or amplitude) from the hypothalamus [21, 25]; iii) suppression of LH pulsatile release from the pituitary [26]; and/or iv) prevention of the ability of the surge-generating mechanism to respond to the preovulatory increases in plasma estradiol concentrations [27, 28]. The first three mechanisms could potentially deprive the ovarian follicle from the necessary gonadotropin drive, thereby blocking the preovulatory estradiol increase; however, the fourth mechanism could involve inhibition of ERα cell activation at critical times. Studies carried out in rats have established that SST is one of the most potent inhibitors of electrical excitability of GnRH neurons identified thus far [29] and inhibits the LH surge when administered centrally [30]. Furthermore, hypothalamic SST release and gene expression are increased during different types of stress such as immobilization [31], hypoxia [32] and acute inflammation [33]. It is, therefore, possible

that SST cells are activated *via* an unknown mechanism to mediate stress-induced disruption of the LH surge.

In the present study, we examined brain tissue of intact ewes sacrificed at various times during the follicular phase with or without the administration of LPS. Our aims were to map the activation patterns of cells containing ERα or SST (by measuring co-localization with c-Fos) in the ARC, VMN and mPOA of control animals, and correlate this with a) peripheral plasma progesterone and estradiol concentrations, and b) with the exhibition of sexual behavior and/or the initiation of an LH surge. Furthermore, we sought to determine whether the disruption of the surge mechanism after LPS involves alteration of ERα or SST cell activation in the ARC, mPOA and VMN as well as describing the temporal relationships between these changes and alterations in plasma steroid concentrations.

MATERIALS AND METHODS

Animals, study design, tissue collection, blood collection and hormone assays.

All procedures were conducted in accordance with requirements of the UK Animal (Scientific Procedures) Act, 1986, and approved by the University of Liverpool Animal Welfare committee. The study was carried out on mature intact Lleyn crossbred ewes in the mid breeding season (6 groups of 4-6 ewes per group). Frozen coronal sections (40 μm) were obtained from the same tissue blocks as described in an earlier study on kisspeptin and corticotropin releasing factor receptor, where full experimental details appear [24]. Briefly: after follicular phase synchronization, ewe and ram estrous behavior was monitored for a 30-min observation period before each blood sample collection at 0 h (progesterone intravaginal device withdrawal; PW), 16 h, 24 h and subsequently at 2 h intervals till 40 h. It was noted when a ewe was within one meter of a ram [behavioral scan sampling; [34] . In addition, the following behavioral signs of estrus were counted: ram nosing perineal region of ewe; ewe being nudged by ram without ewe moving away; and, mounting of ewe by ram without ewe moving away. Due to the 2-hourly observation regime, the beginning/end of a period was respectively defined as the

first/last (minus/plus 1.0 h) 30-min observation period the animal exhibited a particular behavioral sign. Frequent blood sampling, as well as the administration of all substances, was facilitated by insertion of a silastic catheter (Medical grade silastic tubing, internal diameter 1.01 mm, Dow Corning, Reading, UK) into the jugular vein of each ewe under local anesthesia before progesterone withdrawal. Blood samples were collected and centrifuged immediately at 1000 *g* for 20 min at 4^0C. Plasma was stored at -20^0C until analysis. Duplicate samples were analyzed by Enzyme-Linked Immunosorbent Assays (ELISAs) for LH, pregnane metabolites (equivalent to, and hereafter referred to as, progesterone) or cortisol. LH results were expressed as ng equivalent of NIAMDD ovine LH 21 per ml plasma. Estradiol was measured by radioimmunoassay (RIA) using 0.5 ml plasma extracted with 3 ml diethyl ether followed by evaporation to dryness. All assays had been verified for use in sheep [22]. Contemporary inter-assay and intra-assay coefficients of variation for LH, progesterone, cortisol and estradiol were all less than 12%. The minimum detectable amounts were 0.02 ng/ml; 0.16 ng/ml, 0.8 ng/ml and 0.2 pg/ml and assay precisions (in the mid-range of the standard curve) were 0.1 ng/ml, 0.01 ng/ml, 0.2 ng/ml and 0.2 pg/ml, respectively. All samples from individual animals were measured in the same assay for each hormone.

A group of ewes was killed at 0 h (0 h control group; n=5) and another group at 16 h after progesterone withdrawal (16 h control group; n=4). At 28 h, the remaining animals received 2 ml saline vehicle or endotoxin (lipopolysaccharides from *E. coli* 055:B5, LPS, Sigma-Aldrich, UK; i.v. dose of 100 ng/kg body weight). The timing of treatment was chosen in order to precede all sexual behaviors and not just mounting [22]. The dose of LPS is routinely used in our studies and evokes a robust cortisol response and delayed LH surge, with minimal clinical signs of occasional coughing. Two groups were killed at 31 h (31 h control, n=6 and 31 h LPS group, n=5) and two groups at 40 h after PW (40h control, n=5 and 40h LPS group, n=5). Ewes were euthanized with 20 ml 20% w/v sodium pentobarbitone (Pentobarbital, Loveridge, Southampton, UK), containing 25,000 IU heparin and the heads perfused with 2 liters 0.1 M phosphate buffer (PB; pH7.4) containing 25,000 IU per liter of heparin and 1%

sodium nitrate; then 2 liters Zamboni fixative (4% paraformaldehyde and 7.5% saturated picric acid in 0.1 M PB, pH7.4); followed by 500 ml of the same fixative containing 30% sucrose. Hypothalamic blocks (17 mm in width) were obtained (extending from the optic chiasma to the mammillary bodies). Free-floating sections were stored in cryoprotectant solution and stored at -20^{0}C until processed for immunohistochemistry.

ERα and c-Fos dual-label immunofluorescence

For ERα/c-Fos analysis, a series of sections from the mPOA (at the level of the organum vasculosum of the lamina terminalis (OVLT) and the MBH (containing ARC and VMN) were processed for dual-label immunofluorescence. All steps were performed at room temperature unless otherwise stated. Antibodies were diluted with 2.5% normal donkey serum (catalogue item S2170, Biosera, UK), 1% Triton X-100 (T9284, Sigma-Aldrich, Poole, UK) and 0.25% sodium azide (Sigma-Aldrich) in 0.1 M phosphate buffer saline, pH 7.2 (PBS). Free-floating sections were washed thoroughly in PBS for 2 h to remove the cryoprotectant solution followed by 1 h incubation in blocking solution (10% donkey serum in PBS). This was followed by 72 h incubation at 4^{0}C with a mixture of polyclonal rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA) at a dilution of 1:5000 along with monoclonal mouse anti-ERα (clone ID5, M7047, Dako, Carpinteria, CA, USA) at a dilution of 1:50. The c-Fos [35] and ERα [36] antibodies had been validated for use in ovine neural tissue. After incubation with the primary antisera, sections were washed thoroughly and incubated with a mixture of donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch, West Grove, PA) and donkey anti-mouse DyLight 488 (715-485-151, Jackson Immunoresearch, West Grove, PA), both diluted 1:500 for 2 h. Thereafter, sections were washed with PBS followed by a final wash with double-distilled water, mounted on chrome alum gelatine coated slides and cover-slipped with Vectashield anti-fading mounting medium (Vector Laboratories Ltd, UK, H-1000). Negative controls that omitted one of the

primary antibodies completely eliminated the appropriate fluorescence without noticeably affecting the intensity of the other fluorescent probe.

SST and c-Fos dual-label immunofluorescence

For SST/c-Fos analysis, a series of sections from the MBH (containing ARC and VMN) were processed for dual-label immunofluorescence. The protocol was similar to that described above, only this time the primary antibodies were applied sequentially. The polyclonal rabbit anti-c-Fos antibody was followed by washes and incubation for 2 h with donkey anti-rabbit Cy3, diluted 1:500. A second immunofluorescence procedure was then performed, as described above, to localize the second primary antibody: rabbit anti-somatostatin-14 serum (T-4103, Peninsula Laboratories, San Carlos, CA, at a dilution of 1:500), incubated for 72 h at 4^0C and then visualized using donkey-anti-rabbit Dylight 488 (715-485-152, Jackson Immunoresearch, West Grove, PA) at a dilution of 1:500. Thereafter, sections were washed with PBS and mounted on chrome alum gelatin-coated slides and cover-slipped with Vectashield anti-fading mounting medium. The somatostatin-14 antibody was validated for use in ovine neural tissue in Robinson et al., [20]. Negative controls as above were included in each staining run.

Sections were examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20× objective. Photographs were acquired with an image analysis program AxioVision (Zeiss Imaging Systems) and consisted of single ERα or SST staining and single c-Fos staining, as well as a merged image to produce a spectral combination of green (fluorescein) and red (rhodamine) that resulted in identification of dual labeled cells. The areas examined were (as defined by Welento *et al.*, [37]): ARC (3 photographs per section, 3 sections per ewe, which consisted sections from the middle and caudal divisions of the nucleus), mPOA (at the level of the OVLT, 2 photographs per section, 3 sections per ewe) and VMN (4 photographs per

section, 3 sections per ewe). All photographs were imported into Image J version 1.42q, where counts were performed using the cell count plug-in. Initial counts were carried out on the merged images and co-localization was confirmed by flipping through images of the individual c-Fos and ERα or SST micrographs and visually identifying cells that contained both c-Fos label and ERα or SST label with respect to microscopic tissue landmarks. The observer was unaware of the animal identity and group.

Data analysis

The mean total number and percentage of single- or dual-labeled cells was summed from the photographs of each area/section and then averaged for each ewe and compared with GLM ANOVA, followed, where appropriate, by Tukey's multiple comparison *post hoc* tests. Mean (± SEM), as presented in Figures and Results, was calculated by averaging each value for individual animals in each group. Regression analysis was used to examine the association between the percentage of change from 0 h to the two mean consecutive lowest or highest progesterone or estradiol values, respectively, and the percentage of ERα or SST cells that co-localized c-Fos in each area in control animals.

RESULTS

Two animals exhibited estrus and were mounted by a ram within 28 h after PW (i.e., before the predetermined time of treatment; one from each of the 31 h LPS and 40 h LPS groups). The data from these two ewes were excluded from further analyses. None of the animals showed any signs of illness, with a few exceptions of mild coughing and briefly increased respiration rate for the ewes that received LPS.

Behavioral and plasma hormone profiles.

Behavior and plasma hormone profiles have been previously published; for convenience, a summary of results is presented here, however, the reader is directed to Fergani et al., [24] for the full data. In brief, there was no sexual behavior or LH surge recorded in control ewes killed at 0 and 16 h. Eight of eleven control animals, killed at 31 or 40 h, began exhibiting sexual behavior at 28.5 ± 2.4 h after progesterone withdrawal (PW), and three of five ewes in the 40 h control group had an LH surge with a mean onset at 36.7 ± 1.3 h after PW. From the 31 and 40 h LPS groups, only three of eight treated animals exhibited sexual behavior onset at 29.0 ± 2.5 h after PW, and none of the LPS treated ewes exhibited an LH surge within the 40 h of study [24]. Consequently, data were analyzed in two ways: the first consisted of only control ewe data, grouped according to time after PW, and incorporating sexual behavior status and whether an LH surge had occurred; i.e., those killed: at 0 or 16 h after PW; at 31 h after PW but before the onset of sexual behavior (Before sexual behavior, n=3); at 31 or 40 h after PW and during exhibition of sexual behavior but before an LH surge (During sexual behavior, n=5); or after the onset of sexual behavior and during the LH surge (Surge, n=3). This grouping was used to pinpoint the location of ERα cells involved in sexual behavior and/or GnRH/LH surge generating mechanisms in control animals. Secondly, control and treated animal data were grouped according to time of killing after PW, and these data were used to compare treatment effects.

Plasma concentrations of estradiol, progesterone and cortisol have been previously presented [24]. In brief, control plasma estradiol concentrations increased from 28 h after PW to maximum values just before the LH surge onset; 12.2 ± 1.8 pg/ml. However, treatment with LPS decreased estradiol concentrations 8 h after LPS administration (from 11.6 ± 1.6 to 6.9 ± 1.8 pg/ml) and concentrations remained low until ewes were killed at 40 h. Plasma progesterone concentrations decreased from 0 to 28 h after PW in all groups (from 33.7 ± 2.0 to 6.6 ± 0.4 ng/ml). However, LPS treatment increased progesterone concentrations from a mean of 6.9 ± 1.0 ng/ml before treatment to a maximum of 9.9 ± 1.6 ng/ml 2 h after treatment. In all control animals, mean plasma cortisol concentrations remained

low throughout the study (10.5 ± 0.7 ng/ml). However, LPS treatment increased cortisol concentrations to a mean maximum of 157 ± 19.8 ng/ml 2 h after treatment.

Control ewes grouped according to sexual behavior and an LH surge.

ERa/c-Fos in the ARC, mPOA and VMN and association with estradiol and progesterone plasma concentrations.

Photomicrographs of sections dual-labeled for ERα and c-Fos from the mPOA in control ewes are exemplified in Fig.1 A-F. The percentage of ERα neurons that co-localized c-Fos (% ERα/c-Fos) in the ARC increased two-fold in the 'Before sexual behavior', 'During sexual behavior' and 'Surge' groups compared to 0 and 16 h groups ($P<0.05$, for all comparisons; Fig. 2A). In the mPOA, %ERα/c-Fos sequentially increased from 0 h towards 'Before sexual behavior', to 'During sexual behavior' and reached a maximum in animals in the 'Surge' group ($P<0.05$, for all significant comparisons in Fig. 2B). In the VMN, %ERα/c-Fos gradually decreased from 0 h until the 'Before sexual behavior' group ($P<0.05$; Fig. 2C) and then suddenly increased ten-fold (compared to 'Before sexual behavior') in animals 'During sexual behavior' and 'Surge' ($P<0.05$; Fig. 2C).

Using regression analysis, %ERα/c-Fos was variably associated with the percentage change in estradiol concentration between 0 h and the mean two consecutive highest plasma estradiol values. %ERα/c-Fos was not associated with estradiol concentrations in the ARC (P=0.7; Fig. 2D) but was positively associated in the mPOA (P=0.001, RSq=51.1%; Fig. 2E) and the VMN (P=0.02, RSq=21.1%; Fig. 2F).

%ERα/c-Fos was associated with percentage change in progesterone concentration between 0 h and the mean two consecutive lowest plasma progesterone values. %ERα/c-Fos was negatively associated with progesterone concentrations in the ARC (P=0.001, RSq= 64.1 %; Fig. 2D) and the mPOA (P=0.001, RSq=51.1 %; Fig. 2E) but not the VMN (P=0.1; Fig. 2F).

SST/c-Fos in the ARC and VMN and association with estradiol and progesterone plasma concentrations.

The percentage of somatostatin cells that co-localized c-Fos (%SST/c-Fos) in the ARC and the VMN was greatest in the 'Surge' group compared to other stages in the follicular phase ($P<0.05$ for both; Fig. 3A and B).

%SST/c-Fos in the ARC and VMN was positively associated with the percentage change in concentration from 0 h to the mean two consecutive highest plasma estradiol values (ARC: P<0.001, RSq=69.1%; VMN: P<0.001, RSq=77.7%, respectively; Fig. 3C and D). %SST/c-Fos in the ARC and VMN was not associated with the percentage change in concentration from 0 h to the mean two consecutive lowest plasma progesterone concentrations (ARC: P=0.08, VMN: P=0.07, respectively; Fig. 3C and D).

Comparison of control and LPS treated ewes.

ERα/c-Fos in the ARC, mPOA and VMN

Photomicrographs of sections dual-labeled for ERα and c-Fos from the ARC in ewes with or without LPS treatment are exemplified in Fig. 4A-F. The mean total numbers of ERα containing cells in the ARC, mPOA and VMN during the follicular phase in control ewes and after treatment are shown in Table 1.

In the ARC, %ERα/c-Fos in controls increased at 31 h and remained high at 40 h, a time when the majority of control animals were having an LH surge ($P<0.001$ for both; compared to 0 and 16 h control groups, Fig. 5A). However, at 31 h after PW (i.e., 3h after LPS administration), %ERα/c-Fos was markedly lower in the LPS group ($P<0.001$) compared to controls (Fig. 5A). The effect of LPS was still evident between the control and LPS groups at 40 h after PW, (i.e., 12 h after the initial application of saline or LPS; $P<0.001$ Fig. 5A).

In the mPOA, there was a gradual increase in %ERα/c-Fos, with 31 and 40 h control groups having a higher %ERα/c-Fos compared to 0 and 16 h control groups ($P<0.01$ for all comparisons; Fig. 5B). Again however, at 31 h after PW (i.e., 3 h after LPS administration), %ERα/c-Fos was markedly lower in LPS animals ($P<0.05$; Fig. 5B). The effect of LPS was still evident between the control and LPS groups at 40 h after PW, (i.e., 12 h after the initial application of saline or LPS; $P<0.001$; Fig. 5B).

In the VMN, %ERα/c-Fos increased in control animals at 40 h compared to the 0 and 16 h groups ($P<0.02$; Fig. 5C). Percentages in the 31 h control group varied considerably between animals (this group contained animals before behavior onset as well as during behavior) and, therefore, there was no difference from all other control groups. LPS administration did not affect %ERα/c-Fos in the VMN (Fig. 5C). However, when data from LPS treated ewes were re-calculated according to exhibition of sexual behaviour, there was an increase in %ERα/c-Fos in animals that had begun sexual behaviour compared to those that had not (53.1 ± 12.4% vs. 29.0 ± 6.8%, respectively; $P<0.05$, full data not shown).

SST/c-Fos in the ARC and VMN

The numbers of SST immunoreactive cells in the ARC and VMN during the follicular phase and after LPS treatment are shown in Table 2. Photomicrographs from the ARC and VMN dual-labeled with SST and c-Fos are shown in Fig. 6.

%SST/c-Fos in the ARC and in the VMN were higher at 40 h compared to other times examined in the follicular phase ($P<0.05$; Fig. 6A and D). In the ARC, LPS did not have an effect and results were not different to controls at any time (Fig. 6A). By contrast, in the VMN, at 31 h after PW (i.e., 3 h after LPS administration), %SST/c-Fos increased in the LPS group ($P<0.05$; Fig. 6D). At 40 h after PW (i.e., 12 h after LPS administration), when the majority of animals were having an LH surge, LPS and control groups were not different (Fig. 6D).

DISCUSSION

The present results extend our knowledge concerning the steroidal regulation of sexual behavior and the GnRH/LH surge in the ARC, VMN and mPOA of the ewe. We have demonstrated that the pattern of ERα cell activation varies with time during the follicular phase, as well as between hypothalamic regions. In particular, increased ERα cell activation begins in the ARC and mPOA between 16 h after PW and 6-7 h before the LH surge onset, and then extends to the VMN at the onset of sexual behavior and the LH surge. Furthermore, ERα cell activation in the VMN and ARC coincides with maximum activation of SST cells, indicating that at least some of the activated ERα containing cells during the LH surge may be SST in phenotype. This pattern is disturbed by acute LPS administration in the late follicular phase and is associated with failure to exhibit an LH surge.

Pattern of ERα cell and SST cell activation during the follicular phase of intact control ewes.

Approximately 6-7 h before the expected GnRH/LH surge onset (i.e., at 31 h after PW), there was a marked increase in the percentage of activated ERα neurons in the ARC. This coincided with decreased progesterone and increasing estradiol concentrations in plasma and, therefore, indicates they are associated with estradiol positive feedback; i.e., the activation stage of the GnRH/LH surge mechanism. Furthermore, ERα cell activation was maintained throughout the late follicular phase and during the GnRH/LH surge, indicating that ERα cells in the ARC may also be associated with the transmission and surge secretion phases of the GnRH surge mechanism. Interestingly, the cFos activation pattern of ERα cells in the ARC was correlated with circulating plasma progesterone concentrations but not estradiol. Thus, it appears ERα cells within this area are not activated by estradiol in a dose-dependent manner but may rather ‘perceive’ a threshold of estradiol, and respond by becoming active [10, 38]. Moreover, this requires low concentrations of progesterone in the peripheral circulation.

Several different neurochemical phenotypes containing ERα in the ARC have been identified to date and are potential candidates for ‘perceiving’ the increased estradiol signal and activating the GnRH

surge mechanism. A most striking accumulation of ERα in the ARC of female sheep occurs in kisspeptin cells (95%; [39]). However, we and others have shown that only during the LH surge (and not other times in the follicular phase), there is a simultaneous intense activation of ARC kisspeptin neurons [24, 40] indicating that these cells may be associated with the secretory phase of the GnRH surge mechanism. Thus, it appears that there are other neuropeptide cells activated in the ARC at least 6-7 h before the expected surge onset that are not kisspeptin cells, but contain ERα. In this aspect, kisspeptin neurons in this region co-localize two other neuropeptides important for the control of GnRH secretion: neurokinin B and dynorphin (termed KNDy cells; [41-43]). It is, therefore, possible that activated ERα cells predominantly contain neurokinin B or dynorphin rather than kisspeptin. Other potential cell types that may be involved contain β-endorphin, dopamine, neuropeptide Y (NPY) or SST (see later) [16] (3% to 20% of these contain ERα [17, 36, 44]). In addition, 52-61% glutamate neurons in the MBH and 50% galanin neurons across the ovine hypothalamus express ERα [45, 46]. Cells containing all the above neuropeptides have been implicated in the control of GnRH secretion in the ewe [17, 45-48] and, therefore, are potential candidates for mediating stimulatory effects of steroids on GnRH neurons.

Activation of ERα cells in the mPOA increases gradually, culminating in maximum activation during the surge. Interestingly, we have previously observed a parallel gradual escalation of kisspeptin neuron activation and this could account for the pattern observed with ERα cell activation in the present study [24]. Apart from the 50% kisspeptin cells in the ovine mPOA that contain ERα, other potential candidates are GABA cells (40% co-localization with ERα [49]) and galanin expressing cells (50% co-localization with ERα [46]). In addition, nearly all dynorphin cells in the mPOA contain PR [50] and, therefore, ERα [51]. Furthermore, there was a strong correlation between circulating plasma estradiol (positive) and progesterone (negative) concentrations and the percentage of ERα neurons that were activated, indicating that the mPOA is regulated by ovarian steroids in a dose-dependent manner. However, as estradiol implants in the MBH and not the mPOA of the ewe are able to elicit an LH surge

[15], it is possible that ERα cells in this area are activated indirectly, *via* other estradiol responsive neurons that may originate in the MBH.

The ventrolateral part of the VMN has been identified as the most sensitive site for estradiol action on sexual behavior in the female rat [52-54], sheep [14] and monkey [55]. To date, dopamine (DA) and noradrenaline (NA) have received most attention as major regulators of sexual behavior in the ewe by acting upon unknown cells in the VMN [13, 56]. In the present study, activation of ERα neurons in the VMN initially decreased until just before the onset of sexual behavior after which there was a ten-fold increase in animals exhibiting pre-copulatory behaviors (compared to 'Before sexual behavior'). Furthermore, there was a positive correlation between the cFos-activation pattern in ERα cells of the VMN and circulating estradiol (but not progesterone), providing further evidence that these cells may be involved in mediating estradiol stimulation of sexual behavior. Interestingly, the above results concur with a reciprocal pattern of extracellular DA concentrations in the MBH of OVX ewes: as plasma progesterone decreased after PW, there was an increase in DA followed by an acute decrease after administration of estradiol [13]. Similarly, NA increases transiently in MBH extra-cellular fluid during estrus and following sexual interactions with a male [57]. More detailed investigations into interactions between DA, NA and ERα neurons over this period would be illuminating. As mentioned above, 70% of the total ERα immunoreactive cells in the VMN are SST in phenotype [18] and therefore, it would be of great interest to determine whether SST cells receive input from DA and/or NA cells, constituting a possible mechanism for the control of sexual behavior in the ewe. These potential interactions could also account for the delay in ERα cell activation observed in the VMN compared to the ARC and mPOA as dopaminergic input to ERα cells may inhibit their activation until the onset of sexual behaviour. However, these anatomical and functional studies remain to be performed. Alternatively, the delay in activation of VMN ERα neurons may be a result of their indirect/secondary activation *via* ERα cells located in the ARC. Indeed, projections from the ARC towards the VMN are well documented using retrograde tracing techniques [58].

In the present study, there was an increase in the percentage of activated SST neurons in the ARC and VMN during the LH surge compared to other stages in the follicular phase. Thus, SST neurons in the ARC and VMN appear to be directly or indirectly activated by estradiol (we found a positive correlation between activated SST cells and estradiol plasma concentrations) during the surge secretion phase of the GnRH surge mechanism. In accordance with our data, Scanlan et al., [17] report a similar magnitude increase in ARC and VMN SST activation, 18 h after a surge stimulating estradiol injection (i.e., during the surge) in anestrous ewes. By contrast, in an OVX-hormone replacement ewe model, SST mRNA [19] and c-Fos induction in SST neurons [20] was observed 4 h and 6 h after exposure to surge generating estradiol implants, respectively. The latter two reports implied that SST was activated in the early stages of the surge induction process; however, there was no information concerning the surge in those studies. The reason for this time difference in SST cell activation is not known. It is possible that SST neurons activated in the early stages of surge generation are the 30% SST neurons that contain ERα, while those activated at the time of the GnRH surge belong to the 70% non-ERα containing SST cells.

The finding that SST cells are activated during LH surge secretion is particularly interesting, as central administration of SST attenuates the LH surge in rats [30] and abolishes LH pulsatility and dramatically decreases the mean basal level of LH secretion in the ewe [19]. Together, these observations lead to a hypothesis that SST neurons may be important for termination of the GnRH/LH surge. Alternatively, SST neurons may act as a disinhibiting mediator for GnRH secretion by acting on GABA cells located in the vicinity of mPOA GnRH cell bodies [59]. Indeed, microdialysis revealed lower GABA values in the mPOA prior to the GnRH/LH surge [60]. These hypotheses remain to be tested.

The potential pathway *via* which SST neurons influence GnRH secretion in the ewe is unknown. In mice, approximately 50% of GnRH neurons have SST close contacts [29], whereas Koyoma et al.,

410 [61] reported 35 close contacts between each GnRH neuron and SST fibers in the rat. Furthermore,
411 mRNAs for somatostatin receptors 2, 3 and 4 have been identified in murine GnRH cells [62]. Whether
412 SST acts directly on GnRH neurons or potentially *via* interneurons to influence GnRH secretion in the
413 ewe merits further investigation.

414 *Pattern of ERα and SST cell activation during the follicular phase of intact ewes treated with LPS.*

415 Administration of the immunological stressor LPS during the follicular phase leads to a reduction in
416 plasma estradiol concentrations and delays the LH surge onset by approximately 22 h [22]. Plasma
417 estradiol concentrations decreased 8 h after the administration of LPS [24], whereas a decrease in the
418 percentage of activated ERα neurons, in the ARC and mPOA, occurred sooner (3 h after treatment).
419 These results concur with previous studies indicating that there are at least two mechanisms involved
420 in LPS inhibition of the ovarian cycle: one involving disruption of GnRH/LH pulses and, therefore,
421 reduced estradiol secretion from the ovaries; and the other, preventing the ability of the surge-
422 generating mechanism to respond to the preovulatory increase in estradiol [27, 63]. Here, we extend
423 these observations by showing that the latter mechanism involves inhibition of ERα cells that fail to
424 become activated in the ARC and mPOA. Furthermore, our results show that there is a time difference
425 between the two disruptive mechanisms (decrease of plasma estradiol 8 h after LPS administration;
426 decreased activation of ERα cells within 3 h after LPS), indicating that the regulating factors may be
427 different. In support of this dual regulation, Harris *et al.*, [64] report that prostaglandins secreted after
428 LPS treatment have the ability to attenuate GnRH pulses, but administration of the prostaglandin
429 synthesis inhibitor flurbiprofen did not reverse the LH surge delay observed after application of this
430 stressor [65].

431 In the present study, cortisol increased to maximum concentrations immediately after the
432 administration of LPS (i.e., 2 h after treatment; [24]) and is, therefore, a potential candidate for the
433 immediate inhibition of ERα neurons. Indeed, Pierce *et al.*, [66] and Wagenmaker *et al.*, [67] report

that administration of high doses of cortisol disrupt the positive feedback effect of estradiol to trigger an LH surge. One potential inhibiting pathway is *via* glucocorticoid receptors type II (GRII), which are present in ~ 70% of ERα cells located in the mPOA and ARC [51]. However, studies examining the effects of other types of stressors such as insulin-induced hypoglycemia or a layered psychosocial stress paradigm, both accompanied by endogenous cortisol production, report that administration of the progestin/glucorticoid receptor antagonist RU486 did not reverse the LH surge delay or the attenuation of GnRH pulses [21, 68]. It is possible, that cortisol production during insulin-induced hypoglycemia and psychosocial stress is insufficient for a hypothalamic effect.

We observed an increase in plasma progesterone concentrations after LPS, possibly of adrenal origin [24], however, the timing of maximum values varied considerably between animals, from 2 to 10 h after treatment and, therefore, we cannot determine which mechanism is affected by stress-induced increases in progesterone. But it is noteworthy that progesterone has been implicated in both inhibition of GnRH pulses [69] and blocking of the surge mechanism [70-72].

The effects of LPS were still evident 12 h after treatment, when the percentage of activated ERα neurons in the ARC and mPOA remained at low levels. Taking into consideration that these animals did not have an LH surge at the same time as controls, we conclude that the LH surge disruption in response to an immune/inflammatory challenge in the ewe is accompanied by a lack of ERα neuron activation. This compliments our recent results in which the absence of an LH surge was accompanied by the failure of highly estradiol-receptive kisspeptin neurons to be activated [24]. At the same time, co-localization of corticotropin releasing factor receptor type 2 and kisspeptin was increased (>50% co-localization), indicating that this may constitute a potential inhibitory pathway [24]. The precise mechanism by which ERα cells are inhibited following LPS administration remains to be elucidated, however, it may involve other cells of unknown phenotype located in the ARC, mPOA and VMN, as c-Fos is greatly increased in these areas after LPS administration [24].

Intriguingly, the percentage of ERα neurons that were activated in the VMN was not altered by LPS. Since the majority of ERα neurons in the VMN are SST in phenotype [18], we hypothesized that SST neurons would be activated in response to LPS treatment. Indeed, we observed a three-fold increase in SST activation in the VMN 3 h after LPS administration. There are several hypotheses for the role of SST during stress. First, as mentioned above, SST is a potent inhibitor of GnRH neurons in rats [29] and, therefore, it is possible that SST cells are activated through an unknown mechanism to mediate stress-induced disruption of the LH surge *via* direct or indirect action on GnRH cells. Second, in the rat, acute inflammation induced by LPS inhibits secretion of growth hormone (GH) from the pituitary gland and this suppression is mediated by hypothalamic SST [73]. However, in the rat and sheep, SST neurons from the periventricular region and not elsewhere, project to the ME and form a final common pathway for the regulation of GH secretion from the anterior pituitary [17]. Nonetheless, we cannot exclude the possibility that SST neurons in the VMN could be involved in GH suppression indirectly *via* the activation of periventricular SST neurons.

Activation of SST after application of LPS is unlikely to be mediated by cortisol, as the VMN contains very few glucocorticoid receptors type 2 in sheep [51], and adrenalectomy did not prevent the increase in SST mRNA after LPS in rats [33]. *In vitro* evidence indicates that corticotropin releasing factor (CRF) is involved in activating rat somatostatin cells [74]. Indeed, reciprocal connections have been identified between CRF and SST cells in rats [75]. Whether the same is true in the sheep has not yet been investigated, but could constitute a potential pathway via which stressors attenuate GnRH secretion.

Conclusion

The present findings show that ERα cell activation patterns differ at specific times in the follicular phase, as well as between regions. Based on our observations, we hypothesize that once circulating progesterone concentrations have decreased and estradiol concentrations reach a specific ‘threshold’

value (at least 6-7 h before the expected LH surge onset), ERα cell activation increases in the ARC and remains elevated throughout the LH surge. Activation of mPOA ERα cells increases prior to the surge onset but the pattern of activation is gradual. ERα cells in the VMN are activated later than in the ARC and mPOA, and this coincides with the exhibition of sexual behaviors implying that the VMN may be involved in regulation of behavior. Nonetheless, ERα cell activation was at a maximum during the LH surge in all these areas, indicating a role in estradiol positive feedback and GnRH surge secretion. Furthermore, we have identified some of those cells are probably SST in phenotype. The physiological role of increased SST cell activation in the ARC and VMN during the LH surge in the ewe is not known, however, based on previous anatomical and functional studies we hypothesize that this may be involved in GnRH/LH surge termination. Ewes treated with LPS (a potent activator of the stress axis) during the late follicular phase did not have an LH surge at the same time as controls and this was accompanied by a failure of ERα cell activation but an increase in VMN SST cell activity. The precise role of SST in the stress-induced disruption of the GnRH surge, as well as the phenotype identity of other attenuated ERα cells, requires further investigation.

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705

706 **Fig. 1** Sets of photomicrographs from the mPOA that were dual-labeled for c-Fos containing cells
707 (A,D) and their co-localization with ERα (B, E) in control animals at 40 h after PW (during the surge;
708 A, B, C). Panels on the bottom (C, F) are computer-generated merged images of the two top panels
709 illustrating co-localization of c-Fos and ERα. The right *panels* (D, E, F) are the higher magnifications
710 (Scale bar: 50 μm) of the *boxed areas* shown in the *left panels* (A, B, C; scale bar: 150 μm). Examples
711 of single- and double-labeled cells are marked through the panels with arrows and arrowheads,
712 respectively.

713 **Fig 2.** A,B,C: Mean % (±SEM) ERα cells that co-localized c-Fos (%ERα/c-Fos) in the ARC, mPOA
714 and VMN, respectively, at different stages during the follicular phase of control ewes. Animals are
715 grouped according to time after PW as well as hormonal and behavioral status; i.e., grouped into those
716 killed at 0 and 16 h after PW (n=4-5), those killed before the onset of sexual behavior (Before sexual
717 behavior, n=3), those killed after the onset of sexual behavior but before exhibiting an LH surge
718 (During sexual behavior, n=5) and those killed during sexual behavior and an LH surge (Surge, n=3).
719 Within each panel, differences between the percentages are indicated by different letters on top of each
720 bar (P<0.05). D,E,F: Regression graphs showing the correlation between %ERα/c-Fos in the ARC,
721 mPOA and VMN against the % change from 0 h to the mean two consecutive highest or lowest
722 concentrations of estradiol (o, E_2; dotted line) or progesterone (■, P_4; solid line), respectively.

723 **Fig 3.** Mean % (±SEM) somatostatin cells that co-localized c-Fos (%SST/c-Fos) in the ARC (A) and
724 VMN (B) at different stages during the follicular phase of control ewes as determined by dual-
725 immunofluorescence. Animal groupings are explained in Fig 2 legend. Within each panel, differences
726 between the percentages are indicated by different letters on top of each bar (P<0.05). C and D:
727 Regression graphs showing the correlation between %SST/c-Fos in the ARC and VMN against the %
728 change from 0 h to the mean two consecutive highest or lowest concentrations of estradiol (o, E_2;
729 dotted line) or progesterone (■, P_4; solid line), respectively.

730 **Fig. 4** Sets of photomicrographs from the ARC that were dual-labeled for c-Fos cells (A,D) and their
731 co-localization with ERα (B,E) in control animals at 31 h after PW (A, B, C) as well as 3 h after LPS
732 treatment in the late follicular phase (D, E, F). Panels on the bottom (C, F) are computer-generated
733 merged images of the two top panels illustrating co-localization of c-Fos and ERα. Examples of single
734 and double labeled cells are marked through the panels with arrowheads and arrows, respectively.
735 *Scale bars* = 50 μm. 3V = third ventricle.

736 **Fig 5.** Mean % (±SEM) of activated ERα cells in the ARC, mPOA and VMN at various times during
737 the follicular phase of control and treated ewes. Animals are grouped according to killing time after
738 PW i.e., control ewes at 0, 16, 31 and 40 h (n=4-5 per group; black bars) as well as after LPS at 31 and
739 40 h (n=4 for both times; white bars). Treatment with LPS was at 28 h after PW. Within each panel,
740 differences between percentages are indicated by different letters on top of each bar (P<0.05).

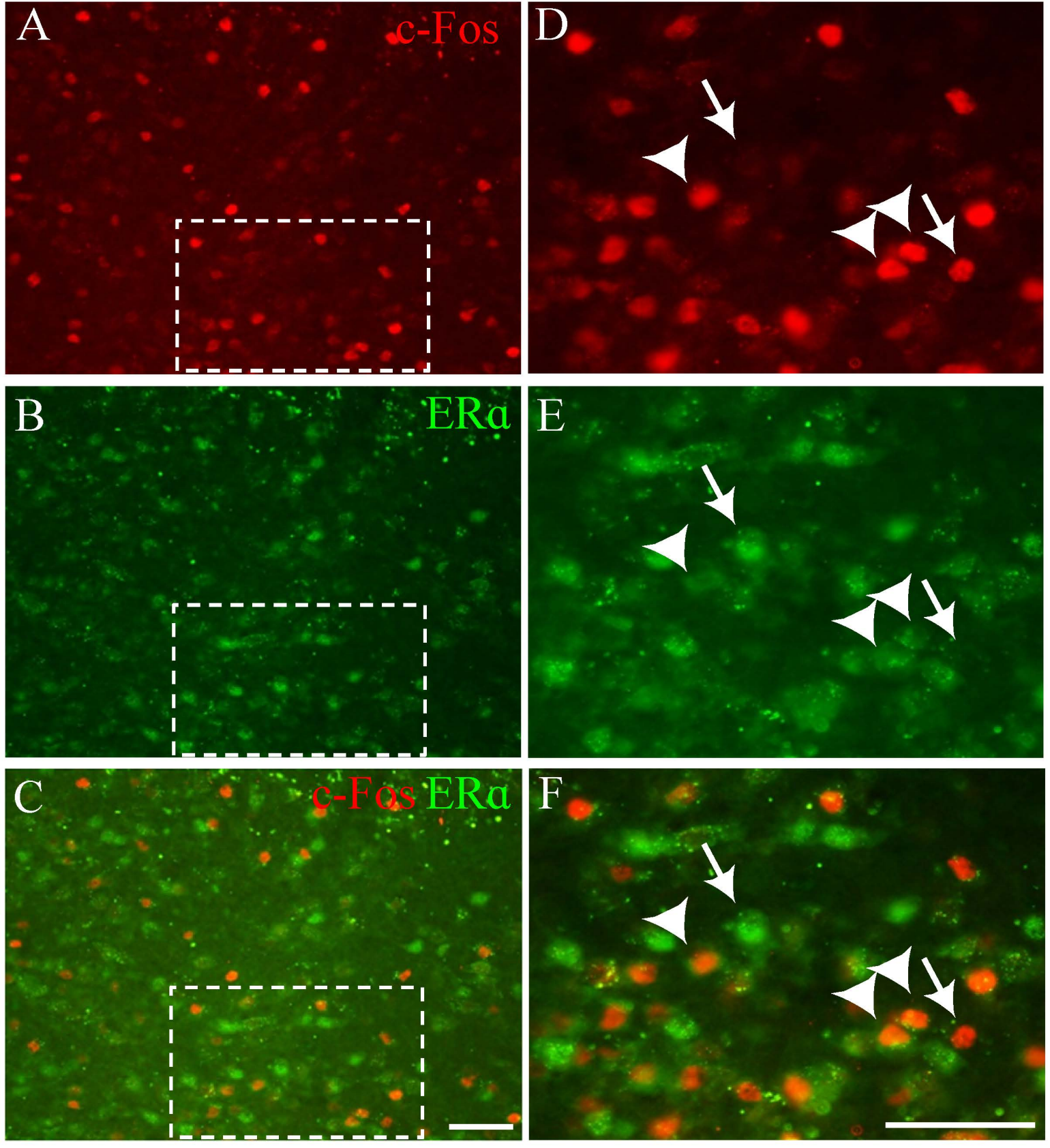
741 **Fig 6.** Mean (±SEM) % of activated SST cells in the ARC (A) and VMN (D) at various times during
742 the follicular phase of control and treated ewes. Animals are grouped according to killing time after
743 PW i.e., control ewes at 0, 16, 31 and 40 h (n=4-5 per group; black bars) as well as after LPS at 31 and
744 40 h (n=4 for both times; white bars). Treatment with LPS was at 28 h after PW. Within each panel,
745 differences between percentages are indicated by different letters on top of each bar (P<0.05). Also
746 shown are photomicrographs from the ARC (B, C) and VMN (E, F) that were dual-labeled with c-Fos
747 and somatostatin in 31 h control ewes (B, E), a 40 h control ewe (during the LH surge; C) and a 31 h
748 LPS treated ewe (F). *White arrows* indicate examples of dual-labeled cells. *Scale bars* = 50 μm. 3V =
749 third ventricle.

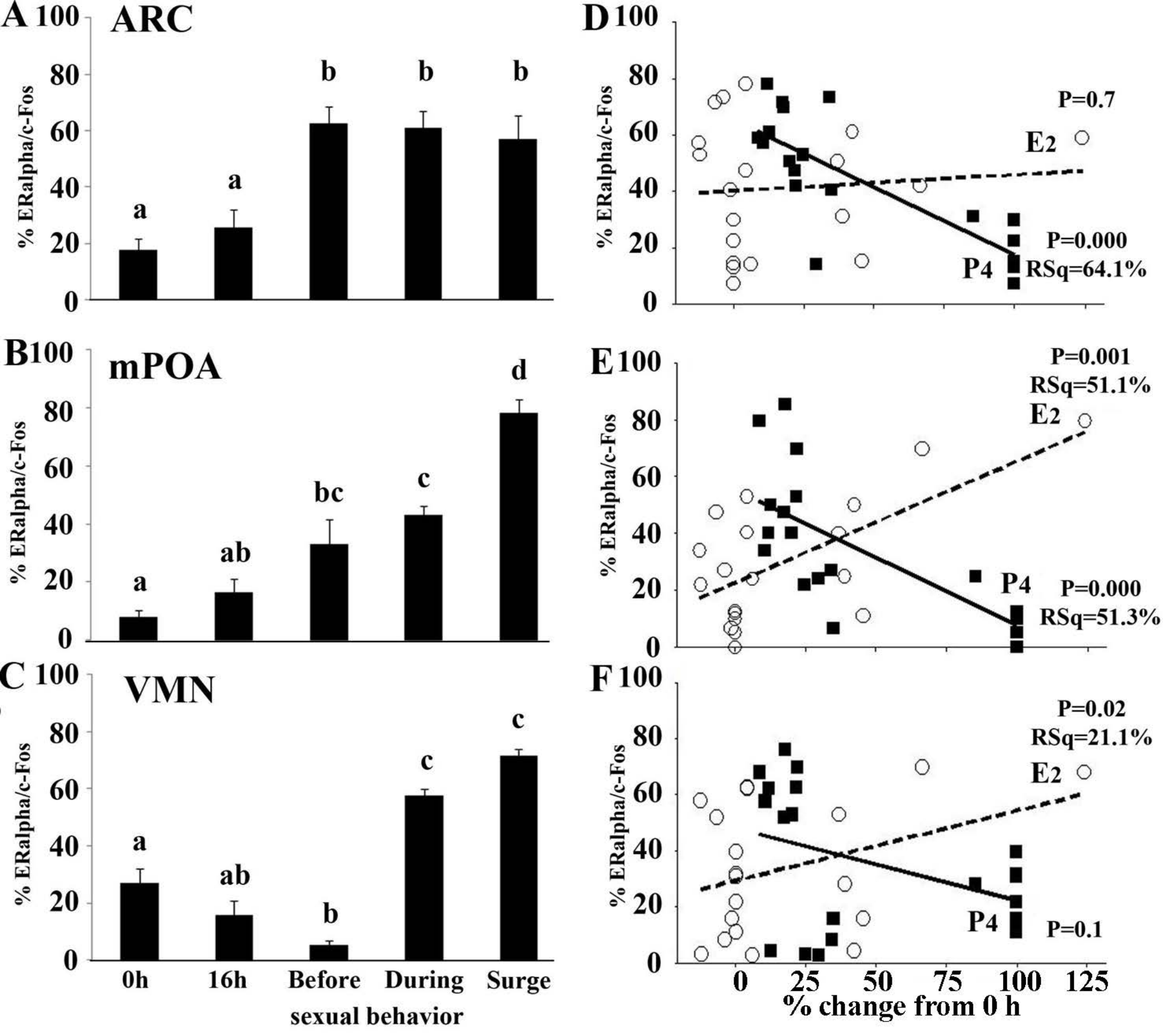
750

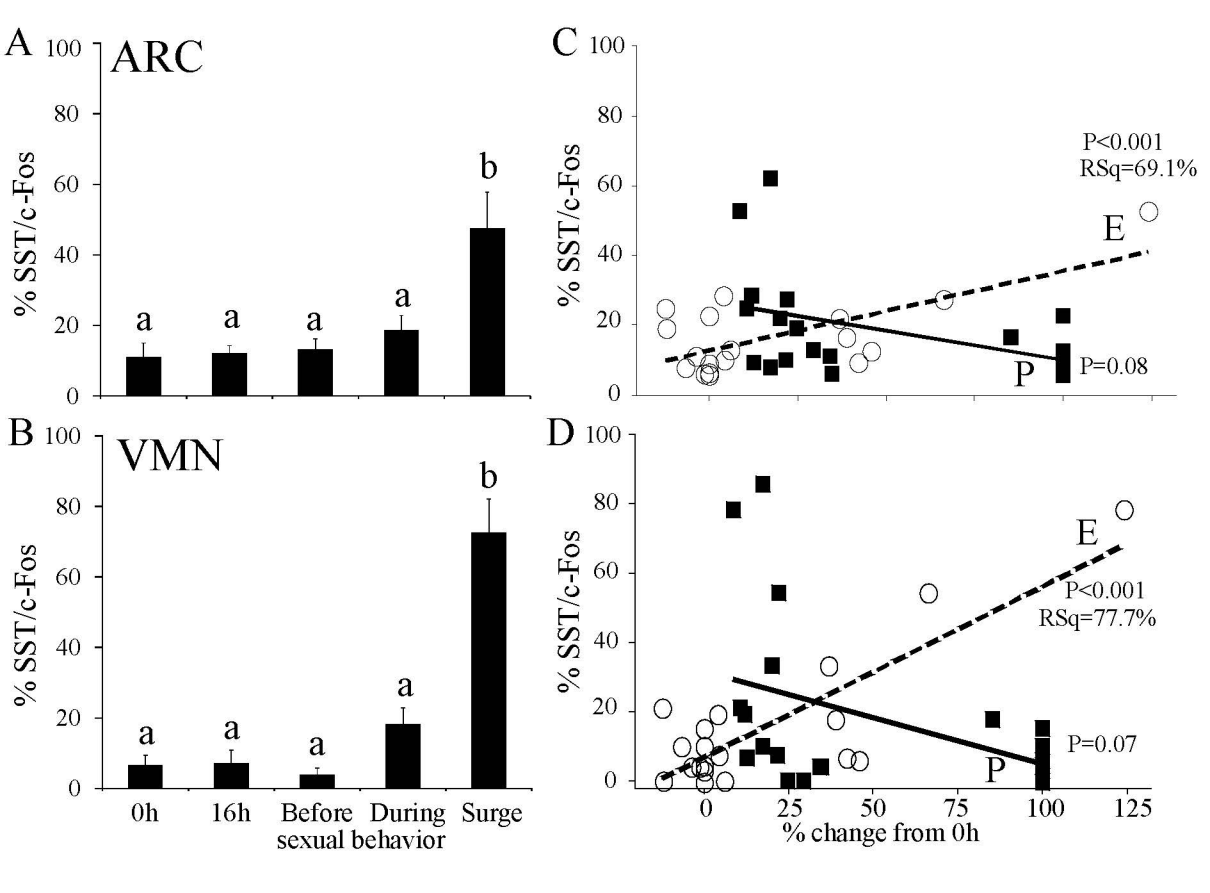
751 **Table 1** Mean (± SEM) total numbers of cells containing ERα in the ARC, mPOA and VMN at
752 different times during the follicular phase, as well as after acute administration of LPS at 28 h during
753 the late follicular phase.

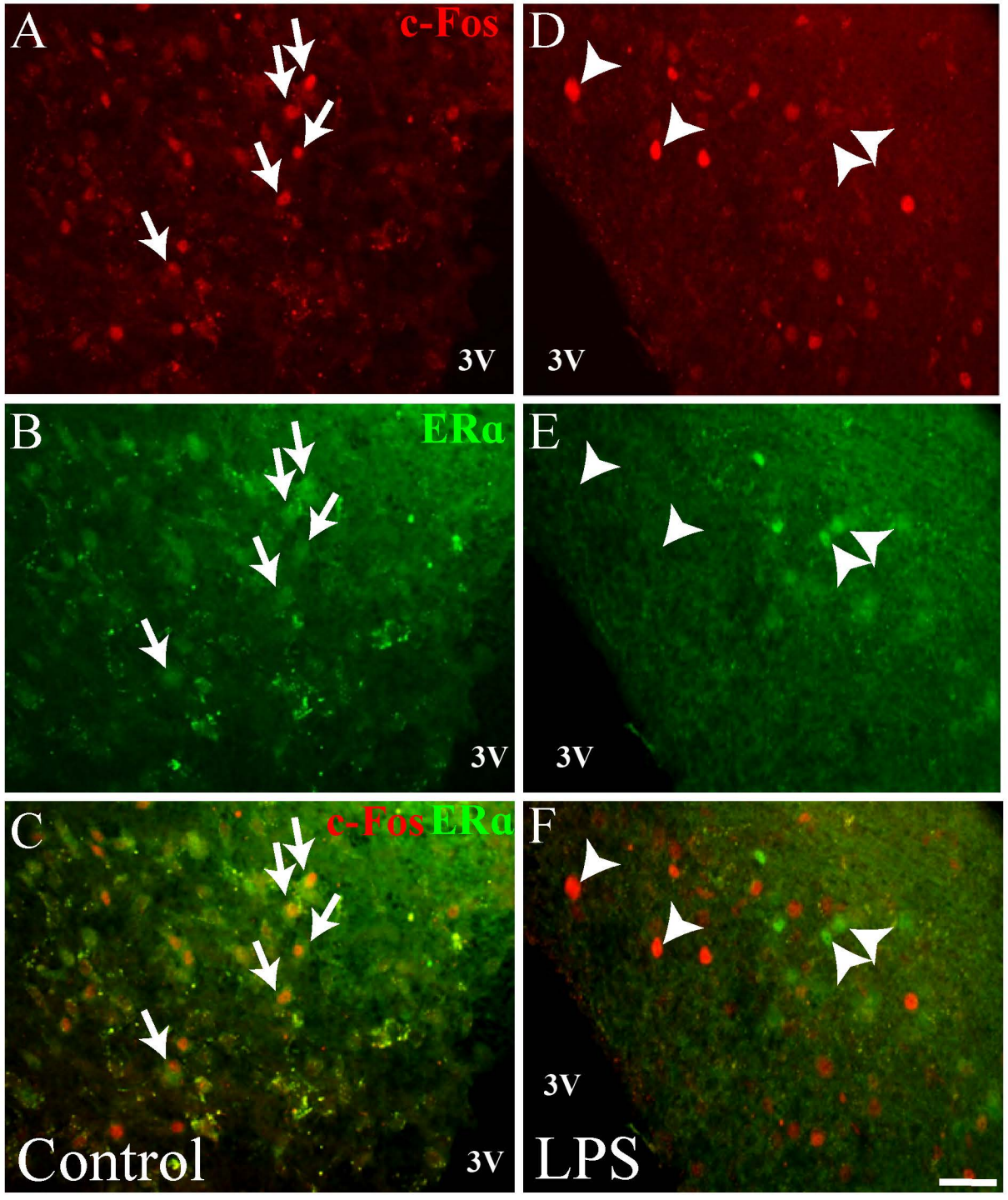
754 **Table 2** Mean (± SEM) total numbers of cells containing somatostatin (SST) in the ARC and VMN at
755 different times during the follicular phase, as well as after acute administration of LPS at 28 h during
756 the late follicular phase.

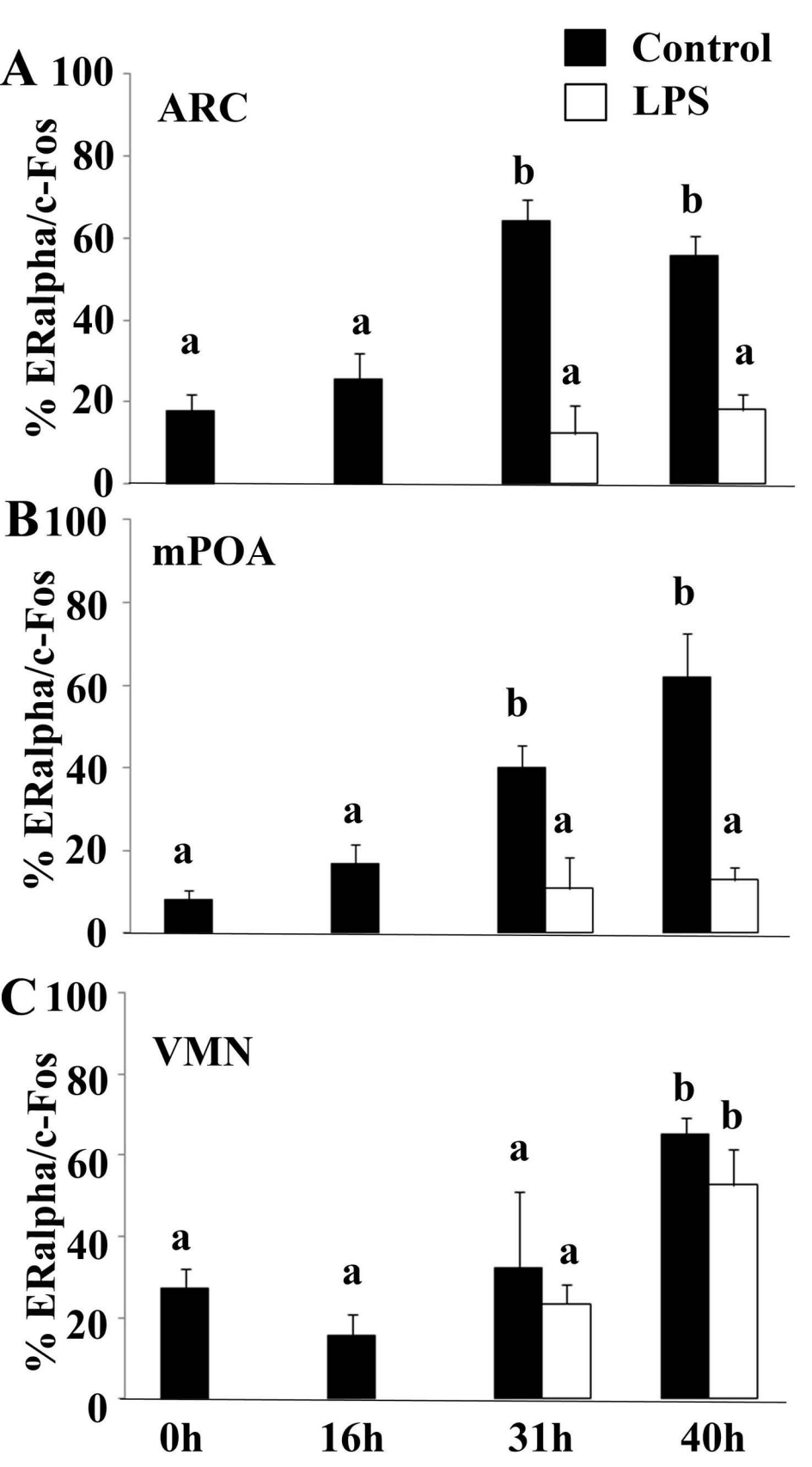
757

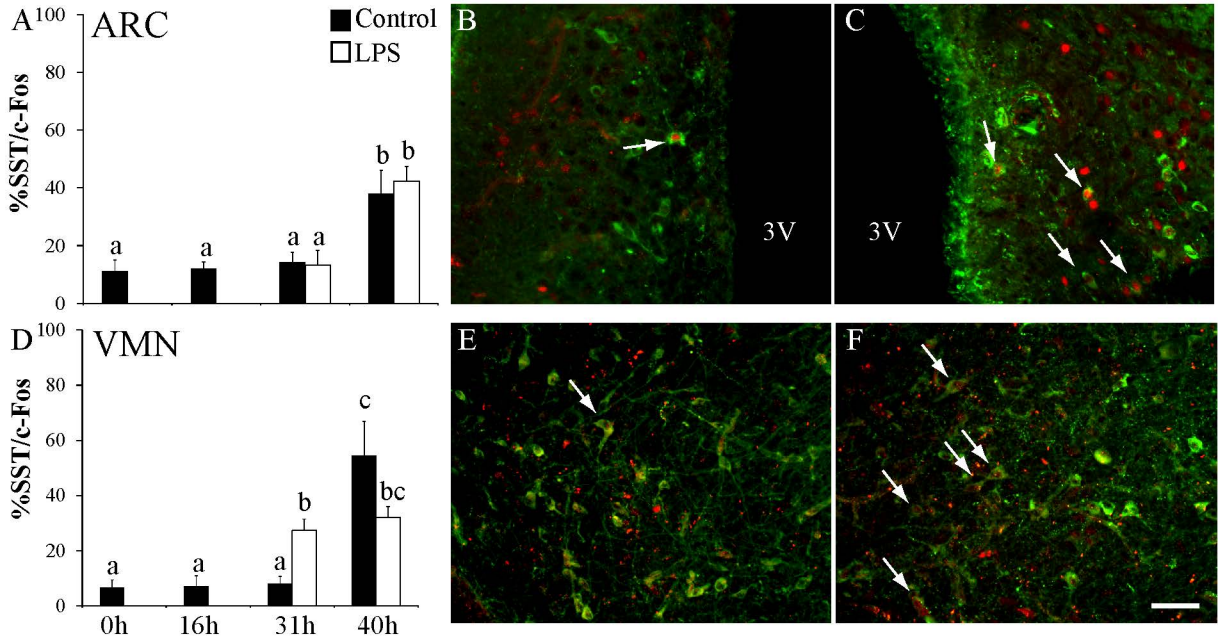

A
c-Fos
D
B
ERα
E
C
c-Fos ERα
F


A
ARC
% ERalpha/c-Fos
a
a
b
b
b
B
mPOA
a
ab
bc
c
d
C
VMN
a
ab
b
c
c
0h
16h
Before
During
Surge
sexual behavior
D
P=0.7
E2
P=0.000
P4
RSq=64.1%
E
P=0.001
RSq=51.1%
E2
P4
P=0.000
RSq=51.3%
F
P=0.02
RSq=21.1%
E2
P4
P=0.1
0
25
50
75
100
125
% change from 0 h


A
ARC
% SST/c-Fos
100
80
60
40
20
0
a
a
a
a
b
B
VMN
% SST/c-Fos
100
80
60
40
20
0
a
a
a
a
b
0h
16h
Before
During
Surge
sexual behavior
C
% SST/c-Fos
100
80
60
40
20
0
P<0.001
RSq=69.1%
E
P
P=0.08
D
% SST/c-Fos
100
80
60
40
20
0
E
P<0.001
RSq=77.7%
P=0.07
0
25
50
75
100
125
% change from 0h


A
c-Fos
3V
D
3V
B
ERα
3V
E
3V
C
c-Fos ERα
Control
3V
F
3V
LPS


A
ARC
B
mPOA
C
VMN
Control
LPS
% ERalpha/c-Fos
100
80
60
40
20
0
a
b
0h
16h
31h
40h


A
ARC
Control
LPS
%SST/c-Fos
100
80
60
40
20
0
a
a
a
a
b
b
B
3V
C
3V
D
VMN
%SST/c-Fos
100
80
60
40
20
0
a
a
a
b
c
bc
0h
16h
31h
40h
E
F

Group	Total number of ERα positive cells		
	Region		
	ARC	mPOA	VMN
0 h	52.3 ± 26.9	15.2 ± 4.1	38.0 ± 8.3
16 h	57.9 ± 19.5	17.2 ± 3.4	49.8 ± 16.4
31 h control	96.6 ± 21.1	39.5 ± 13.0	49.6 ± 14.4
31h LPS	41.3 ± 16.6	37.1 ± 22.5	78.0 ± 12.9
40h control	89.7 ± 19.3	59.0 ± 26.2	100.4 ± 20.8*
40 h LPS	59.9 ± 18.2	26.0 ± 6.6	75.3 ± 12.7

* within columns, $P<0.05$ compared to 0h, 16h, 31h control groups.

Group	Total number of SST positive cells	
	Region	
	ARC	VMN
0 h	48.9 ± 15.1	29.8 ± 9.9
16 h	55.6 ± 16.9	25.9 ± 15.9
31 h control	36.5 ± 10.3	22.3 ± 6.0
31 h LPS	32.1 ± 5.6	28.0 ± 5.7
40 h control	58.1 ± 16.0	36.5 ± 17.2
40 h LPS	52.0 ± 21.1	58.8 ± 18.0