

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 (ER α) mediate estradiol feedback to regulate endocrine and behavioral events during the estrous cycle. A percentage of ER α 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 ER α 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 ER α 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 ER α 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 ER α cells that co-localized c-Fos (%ER α /c-Fos) in the ARC and mPOA from 31 h after PW and throughout the LH surge. In the VMN, %ER α /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 %ER α /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 ER α 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 ER α 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 ER α 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.

53 INTRODUCTION

54 The ovarian steroid hormone estradiol is of central importance in the control of the hypothalamic-
 55 pituitary-gonadal (HPG) axis in female mammals. For the greater part of the ovarian cycle in ewes,
 56 progesterone and estradiol act synergistically to restrain GnRH/LH (gonadotropin releasing
 57 hormone/luteinizing hormone) secretion through a negative feedback. However, during the late
 58 follicular phase, minute-by-minute portal blood sampling in conscious ewes revealed a 'switch' from
 59 inhibition to enhancement of GnRH secretion [1, 2]. This constitutes estradiol positive feedback and
 60 triggers the onsets of GnRH/LH surge secretion. Steroid hormone signals do not impinge directly on
 61 GnRH cells as these cells do not possess progesterone receptors (PR) or estrogen alpha receptors (ER α)
 62 [3-5]. Some GnRH neurons express ER β [6], although it is unlikely that ER β plays a major role in the
 63 feedback regulation of GnRH/LH secretion because ER β knock-out mice have normal fertility [7, 8].

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

72 To date, studies using localized implants have demonstrated that estradiol acts in the mediobasal
 73 hypothalamus (MBH; vicinity of the VMN/ARC) to induce both the surge and sexual behaviors [14,
 74 15]. However, we still don't know the precise location and timing of cell activation within the areas
 75 involved at each stage of the surge- and behavior-generating mechanisms in response to changes in the
 76 steroid hormone milieu. These areas contain several types of neurons, sub-populations of which co-

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

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

125 first/last (minus/plus 1.0 h) 30-min observation period the animal exhibited a particular behavioral
126 sign. Frequent blood sampling, as well as the administration of all substances, was facilitated by
127 insertion of a silastic catheter (Medical grade silastic tubing, internal diameter 1.01 mm, Dow Corning,
128 Reading, UK) into the jugular vein of each ewe under local anesthesia before progesterone withdrawal.
129 Blood samples were collected and centrifuged immediately at 1000 g for 20 min at 4°C. Plasma was
130 stored at -20°C until analysis. Duplicate samples were analyzed by Enzyme-Linked Immunosorbent
131 Assays (ELISAs) for LH, pregnane metabolites (equivalent to, and hereafter referred to as,
132 progesterone) or cortisol. LH results were expressed as ng equivalent of NIAMDD ovine LH 21 per
133 ml plasma. Estradiol was measured by radioimmunoassay (RIA) using 0.5 ml plasma extracted with 3
134 ml diethyl ether followed by evaporation to dryness. All assays had been verified for use in sheep [22].
135 Contemporary inter-assay and intra-assay coefficients of variation for LH, progesterone, cortisol and
136 estradiol were all less than 12%. The minimum detectable amounts were 0.02 ng/ml; 0.16 ng/ml, 0.8
137 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
138 ng/ml, 0.2 ng/ml and 0.2 pg/ml, respectively. All samples from individual animals were measured in
139 the same assay for each hormone.

140 A group of ewes was killed at 0 h (0 h control group; n=5) and another group at 16 h after progesterone
141 withdrawal (16 h control group; n=4). At 28 h, the remaining animals received 2 ml saline vehicle or
142 endotoxin (lipopolysaccharides from *E. coli* 055:B5, LPS, Sigma-Aldrich, UK; i.v. dose of 100 ng/kg
143 body weight). The timing of treatment was chosen in order to precede all sexual behaviors and not just
144 mounting [22]. The dose of LPS is routinely used in our studies and evokes a robust cortisol response
145 and delayed LH surge, with minimal clinical signs of occasional coughing. Two groups were killed at
146 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
147 and 40h LPS group, n=5). Ewes were euthanized with 20 ml 20% w/v sodium pentobarbitone
148 (Pentobarbital, Loveridge, Southampton, UK), containing 25,000 IU heparin and the heads perfused
149 with 2 liters 0.1 M phosphate buffer (PB; pH7.4) containing 25,000 IU per liter of heparin and 1%

150 sodium nitrate; then 2 liters Zamboni fixative (4% paraformaldehyde and 7.5% saturated picric acid in
 151 0.1 M PB, pH7.4); followed by 500 ml of the same fixative containing 30% sucrose. Hypothalamic
 152 blocks (17 mm in width) were obtained (extending from the optic chiasma to the mammillary bodies).
 153 Free-floating sections were stored in cryoprotectant solution and stored at -20°C until processed for
 154 immunohistochemistry.

155 *ER α and c-Fos dual-label immunofluorescence*

156 For ER α /c-Fos analysis, a series of sections from the mPOA (at the level of the organum vasculosum
 157 of the lamina terminalis (OVLT) and the MBH (containing ARC and VMN) were processed for dual-
 158 label immunofluorescence. All steps were performed at room temperature unless otherwise stated.
 159 Antibodies were diluted with 2.5% normal donkey serum (catalogue item S2170, Biosera, UK), 1%
 160 Triton X-100 (T9284, Sigma-Aldrich, Poole, UK) and 0.25% sodium azide (Sigma-Aldrich) in 0.1 M
 161 phosphate buffer saline, pH 7.2 (PBS). Free-floating sections were washed thoroughly in PBS for 2 h
 162 to remove the cryoprotectant solution followed by 1 h incubation in blocking solution (10% donkey
 163 serum in PBS). This was followed by 72 h incubation at 4°C with a mixture of polyclonal rabbit anti-
 164 c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA) at a dilution of 1:5000 along with
 165 monoclonal mouse anti-ER α (clone ID5, M7047, Dako, Carpinteria, CA, USA) at a dilution of 1:50.
 166 The c-Fos [35] and ER α [36] antibodies had been validated for use in ovine neural tissue. After
 167 incubation with the primary antisera, sections were washed thoroughly and incubated with a mixture
 168 of donkey anti-rabbit Cy3 (711-165-152, Jackson ImmunoResearch, West Grove, PA) and donkey anti-
 169 mouse DyLight 488 (715-485-151, Jackson ImmunoResearch, West Grove, PA), both diluted 1:500 for
 170 2 h. Thereafter, sections were washed with PBS followed by a final wash with double-distilled water,
 171 mounted on chrome alum gelatine coated slides and cover-slipped with Vectashield anti-fading
 172 mounting medium (Vector Laboratories Ltd, UK, H-1000). Negative controls that omitted one of the

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

175 *SST and c-Fos dual-label immunofluorescence*

176 For SST/c-Fos analysis, a series of sections from the MBH (containing ARC and VMN) were
177 processed for dual-label immunofluorescence. The protocol was similar to that described above, only
178 this time the primary antibodies were applied sequentially. The polyclonal rabbit anti-c-Fos antibody
179 was followed by washes and incubation for 2 h with donkey anti-rabbit Cy3, diluted 1:500. A second
180 immunofluorescence procedure was then performed, as described above, to localize the second
181 primary antibody: rabbit anti-somatostatin-14 serum (T-4103, Peninsula Laboratories, San Carlos, CA,
182 at a dilution of 1:500), incubated for 72 h at 4⁰C and then visualized using donkey-anti-rabbit Dylight
183 488 (715-485-152, Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:500. Thereafter,
184 sections were washed with PBS and mounted on chrome alum gelatin-coated slides and cover-slipped
185 with Vectashield anti-fading mounting medium. The somatostatin-14 antibody was validated for use
186 in ovine neural tissue in Robinson et al., [20]. Negative controls as above were included in each
187 staining run.

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

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

234 Plasma concentrations of estradiol, progesterone and cortisol have been previously presented [24]. In
 235 brief, control plasma estradiol concentrations increased from 28 h after PW to maximum values just
 236 before the LH surge onset; 12.2 ± 1.8 pg/ml. However, treatment with LPS decreased estradiol
 237 concentrations 8 h after LPS administration (from 11.6 ± 1.6 to 6.9 ± 1.8 pg/ml) and concentrations
 238 remained low until ewes were killed at 40 h. Plasma progesterone concentrations decreased from 0 to
 239 28 h after PW in all groups (from 33.7 ± 2.0 to 6.6 ± 0.4 ng/ml). However, LPS treatment increased
 240 progesterone concentrations from a mean of 6.9 ± 1.0 ng/ml before treatment to a maximum of $9.9 \pm$
 241 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.

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

Photomicrographs of sections dual-labeled for ER α and c-Fos from the mPOA in control ewes are exemplified in Fig.1 A-F. The percentage of ER α neurons that co-localized c-Fos (% ER α /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, %ER α /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, %ER α /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, %ER α /c-Fos was variably associated with the percentage change in estradiol concentration between 0 h and the mean two consecutive highest plasma estradiol values. %ER α /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).

%ER α /c-Fos was associated with percentage change in progesterone concentration between 0 h and the mean two consecutive lowest plasma progesterone values. %ER α /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).

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

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

270 %SST/c-Fos in the ARC and VMN was positively associated with the percentage change in
 271 concentration from 0 h to the mean two consecutive highest plasma estradiol values (ARC: $P<0.001$,
 272 $RSq=69.1\%$; VMN: $P<0.001$, $RSq=77.7\%$, respectively; Fig. 3C and D). %SST/c-Fos in the ARC and
 273 VMN was not associated with the percentage change in concentration from 0 h to the mean two
 274 consecutive lowest plasma progesterone concentrations (ARC: $P=0.08$, VMN: $P=0.07$, respectively;
 275 Fig. 3C and D).

276 *Comparison of control and LPS treated ewes.*

277 *ER α /c-Fos in the ARC, mPOA and VMN*

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

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

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

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

301 *SST/c-Fos in the ARC and VMN*

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

305 %SST/c-Fos in the ARC and in the VMN were higher at 40 h compared to other times examined in
 306 the follicular phase ($P<0.05$; Fig. 6A and D). In the ARC, LPS did not have an effect and results were
 307 not different to controls at any time (Fig. 6A). By contrast, in the VMN, at 31 h after PW (i.e., 3 h after
 308 LPS administration), %SST/c-Fos increased in the LPS group ($P<0.05$; Fig. 6D). At 40 h after PW
 309 (i.e., 12 h after LPS administration), when the majority of animals were having an LH surge, LPS and
 310 control groups were not different (Fig. 6D).

311 **DISCUSSION**

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

321 *Pattern of ER α cell and SST cell activation during the follicular phase of intact control ewes.*

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

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

surge mechanism. A most striking accumulation of ER α 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 ER α . 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 ER α 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 ER α [17, 36, 44]). In addition, 52-61% glutamate neurons in the MBH and 50% galanin neurons across the ovine hypothalamus express ER α [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 ER α 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 ER α cell activation in the present study [24]. Apart from the 50% kisspeptin cells in the ovine mPOA that contain ER α , other potential candidates are GABA cells (40% co-localization with ER α [49]) and galanin expressing cells (50% co-localization with ER α [46]). In addition, nearly all dynorphin cells in the mPOA contain PR [50] and, therefore, ER α [51]. Furthermore, there was a strong correlation between circulating plasma estradiol (positive) and progesterone (negative) concentrations and the percentage of ER α 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

361 [15], it is possible that ER α cells in this area are activated indirectly, *via* other estradiol responsive
362 neurons that may originate in the MBH.

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

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

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

408 The potential pathway *via* which SST neurons influence GnRH secretion in the ewe is unknown. In
409 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 ER α 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 ER α 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 ER α 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 ER α 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 ER α neurons. Indeed, Pierce *et al.*, [66] and Wagenmaker *et al.*, [67] report

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

442 We observed an increase in plasma progesterone concentrations after LPS, possibly of adrenal origin
443 [24], however, the timing of maximum values varied considerably between animals, from 2 to 10 h
444 after treatment and, therefore, we cannot determine which mechanism is affected by stress-induced
445 increases in progesterone. But it is noteworthy that progesterone has been implicated in both inhibition
446 of GnRH pulses [69] and blocking of the surge mechanism [70-72].

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

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

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

478 *Conclusion*

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

value (at least 6-7 h before the expected LH surge onset), ER α cell activation increases in the ARC and remains elevated throughout the LH surge. Activation of mPOA ER α cells increases prior to the surge onset but the pattern of activation is gradual. ER α 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, ER α 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 ER α 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 ER α cells, requires further investigation.

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705

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

Fig 2. A,B,C: Mean % (\pm SEM) ER α cells that co-localized c-Fos (%ER α /c-Fos) in the ARC, mPOA and VMN, respectively, at different stages during the follicular phase of control ewes. Animals are grouped according to time after PW as well as hormonal and behavioral status; i.e., grouped into those killed at 0 and 16 h after PW (n=4-5), those killed before the onset of sexual behavior (Before sexual behavior, n=3), those killed after the onset of sexual behavior but before exhibiting an LH surge (During sexual behavior, n=5) and those killed during sexual behavior and an LH surge (Surge, n=3). Within each panel, differences between the percentages are indicated by different letters on top of each bar ($P<0.05$). D,E,F: Regression graphs showing the correlation between %ER α /c-Fos in the ARC, mPOA and VMN against the % change from 0 h to the mean two consecutive highest or lowest concentrations of estradiol (o, E₂; dotted line) or progesterone (■, P₄; solid line), respectively.

Fig 3. Mean % (\pm SEM) somatostatin cells that co-localized c-Fos (%SST/c-Fos) in the ARC (A) and VMN (B) at different stages during the follicular phase of control ewes as determined by dual-immunofluorescence. Animal groupings are explained in Fig 2 legend. Within each panel, differences between the percentages are indicated by different letters on top of each bar ($P<0.05$). C and D: Regression graphs showing the correlation between %SST/c-Fos in the ARC and VMN against the % change from 0 h to the mean two consecutive highest or lowest concentrations of estradiol (o, E₂; dotted line) or progesterone (■, P₄; solid line), respectively.

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

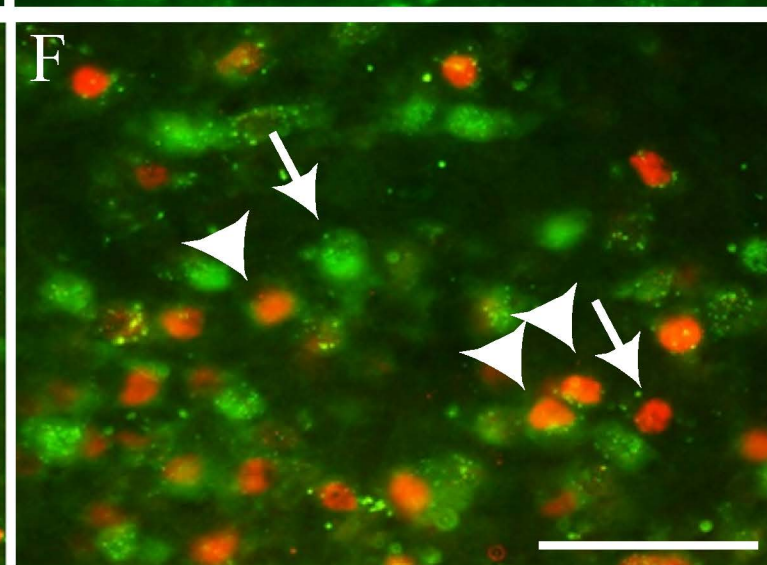
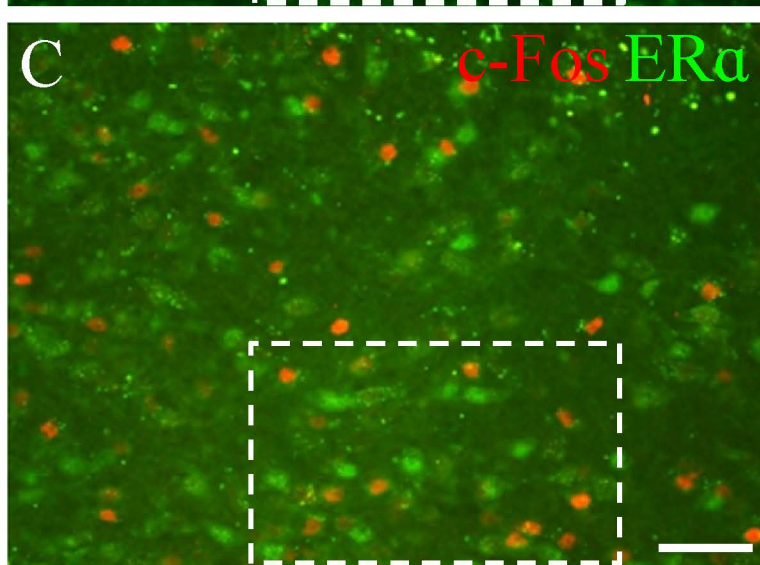
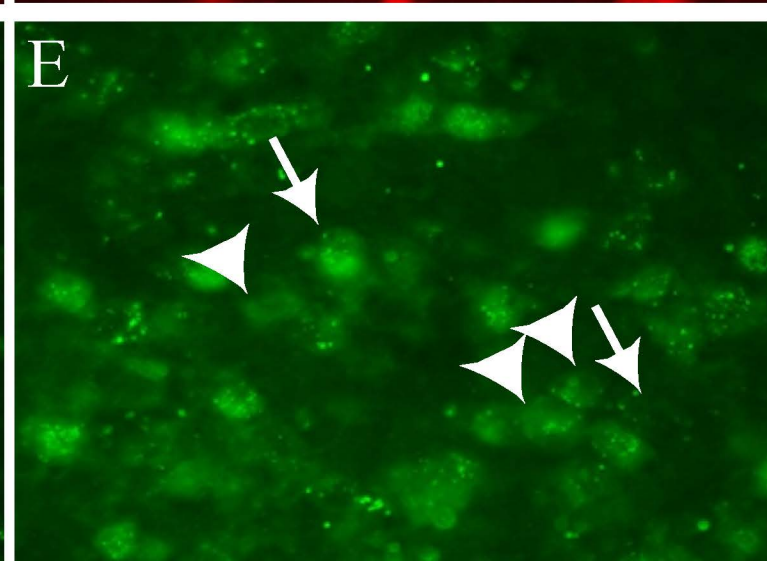
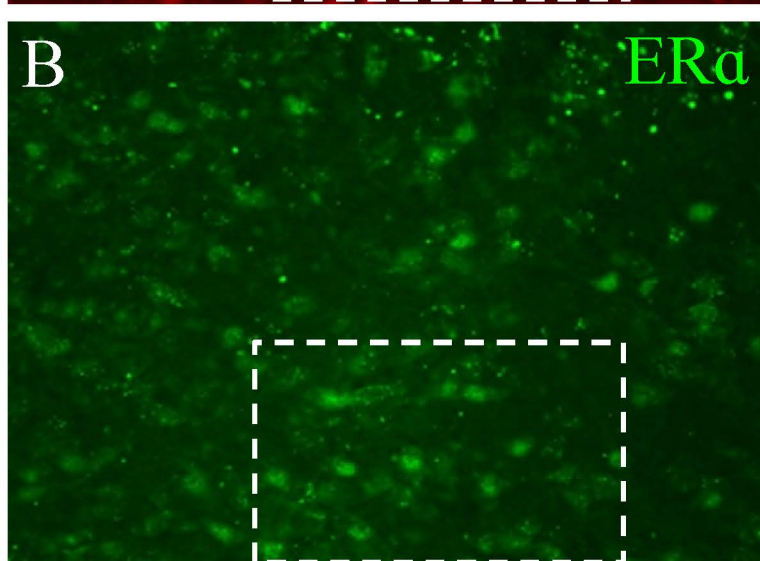
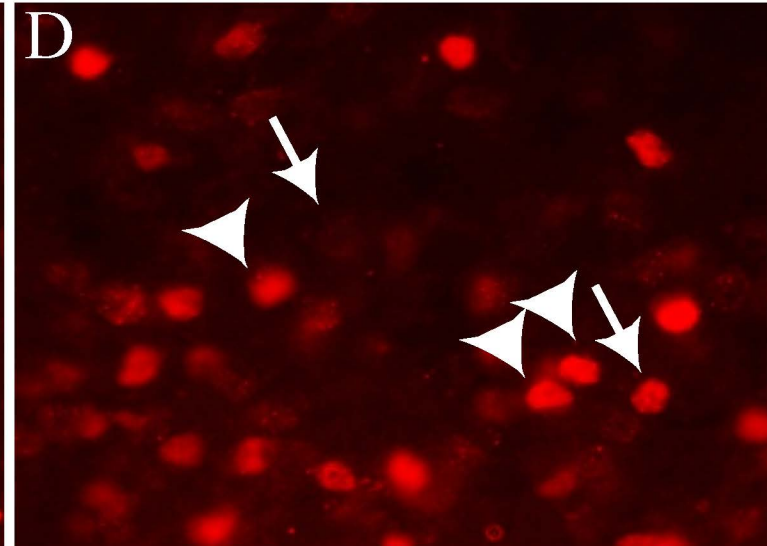
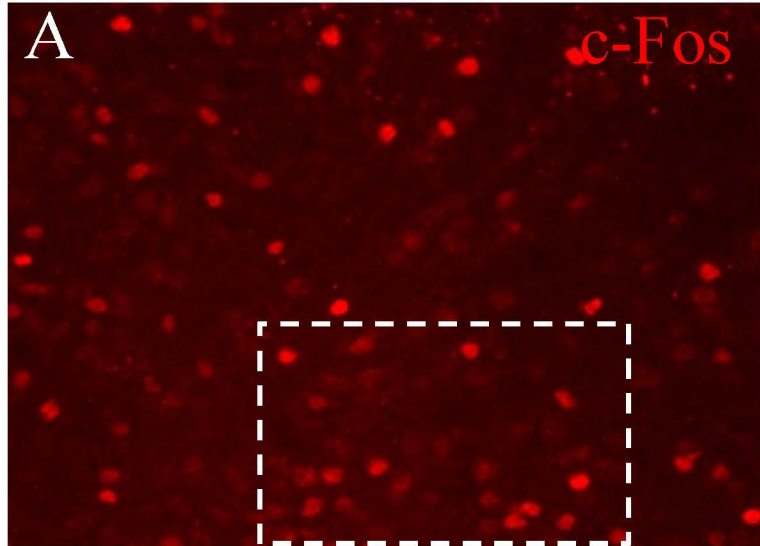
Fig 5. Mean % (\pm SEM) of activated ER α cells in the ARC, mPOA and VMN at various times during the follicular phase of control and treated ewes. Animals are grouped according to killing time after PW i.e., control ewes at 0, 16, 31 and 40 h (n=4-5 per group; black bars) as well as after LPS at 31 and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

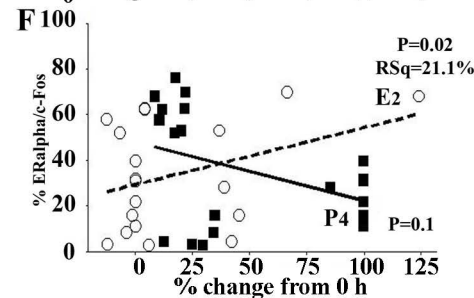
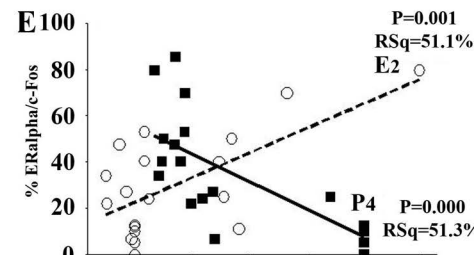
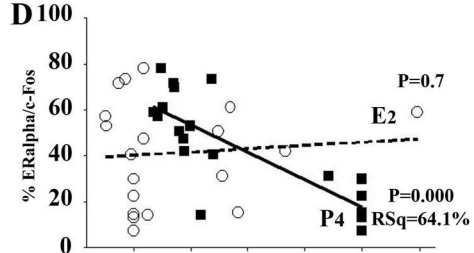
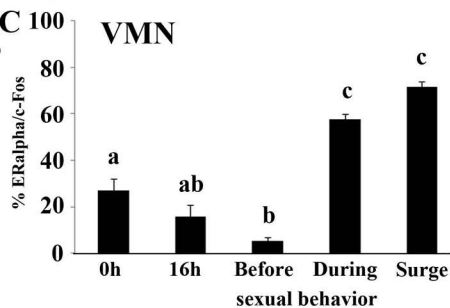
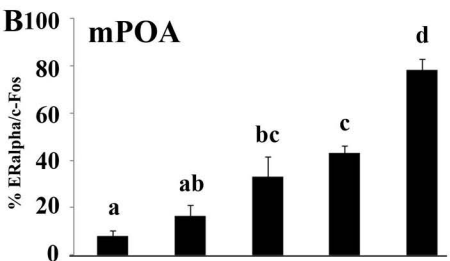
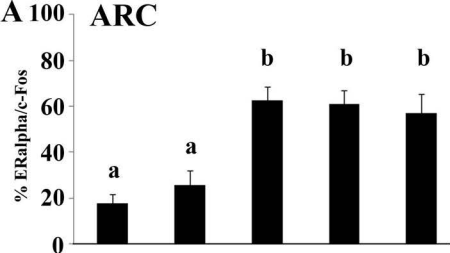
Fig 6. Mean (\pm SEM) % of activated SST cells in the ARC (A) and VMN (D) at various times during the follicular phase of control and treated ewes. Animals are grouped according to killing time after PW i.e., control ewes at 0, 16, 31 and 40 h (n=4-5 per group; black bars) as well as after LPS at 31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

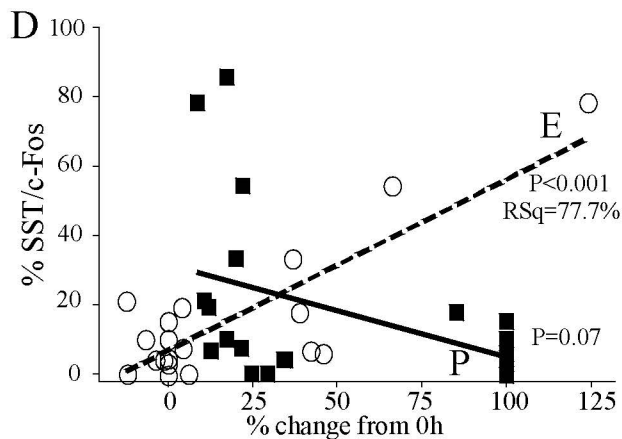
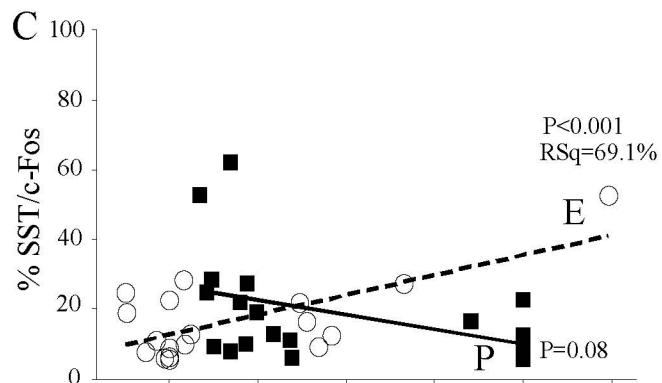
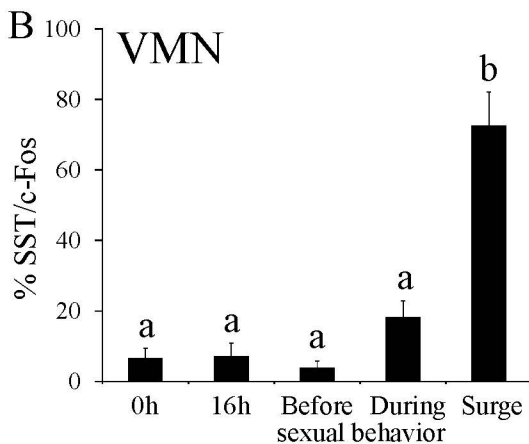
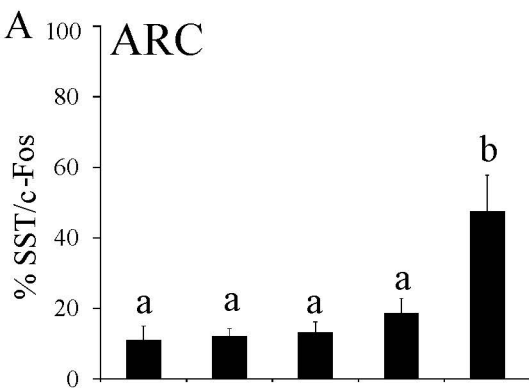
Table 1 Mean (\pm SEM) total numbers of cells containing ER α in the ARC, mPOA and VMN at different times during the follicular phase, as well as after acute administration of LPS at 28 h during the late follicular phase.

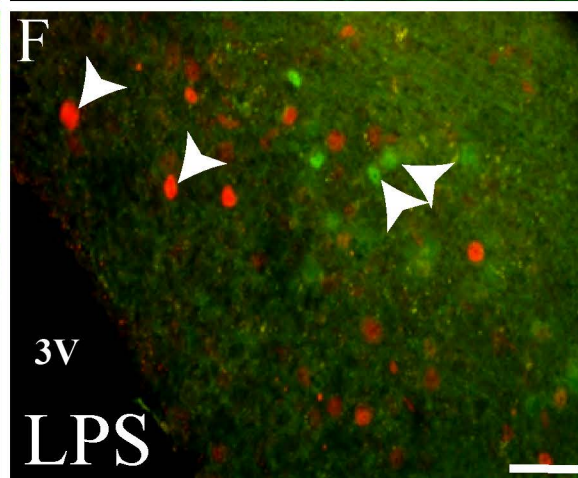
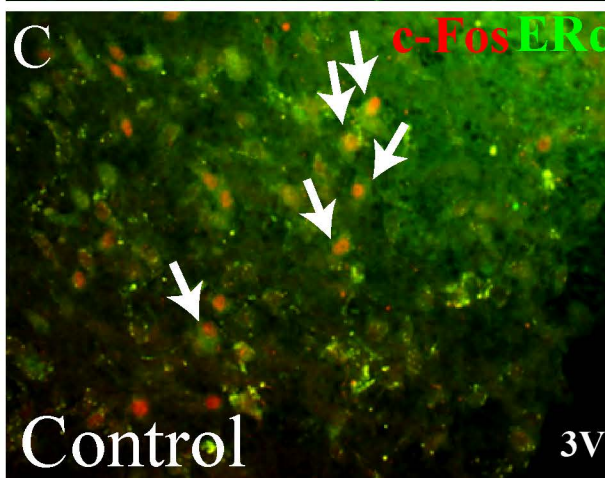
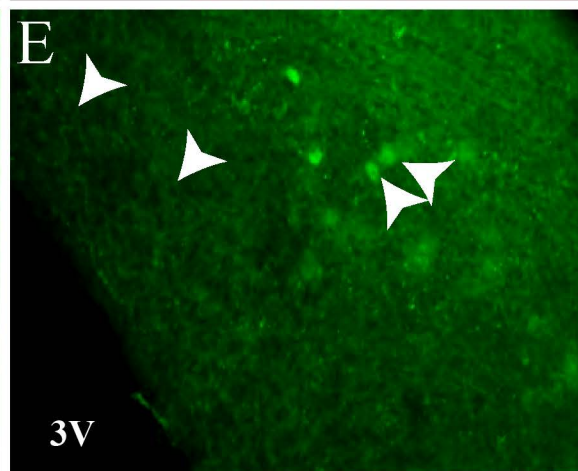
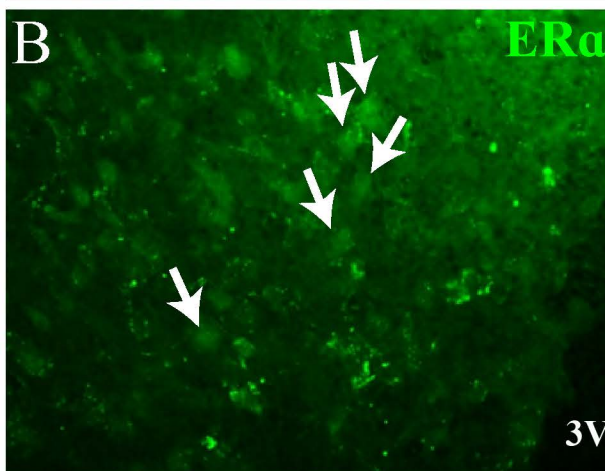
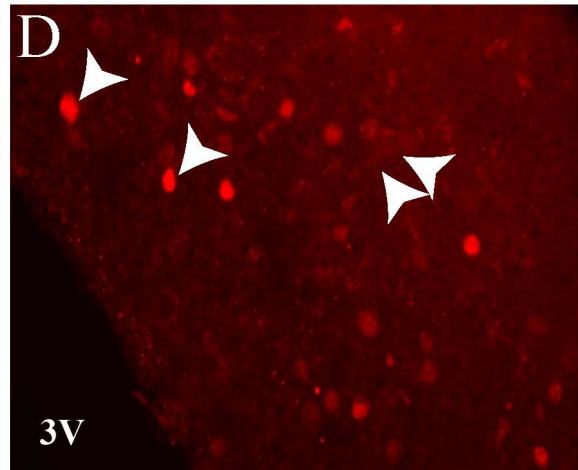
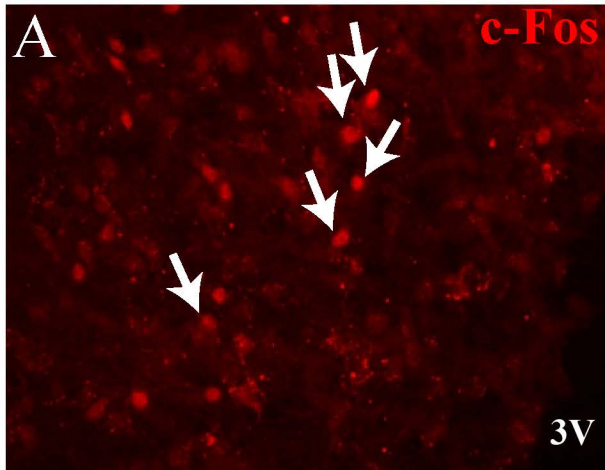
754 **Table 2** Mean (\pm SEM) total numbers of cells containing somatostatin (SST) in the ARC and VMN at
755 different times during the follicular phase, as well as after acute administration of LPS at 28 h during
756 the late follicular phase.

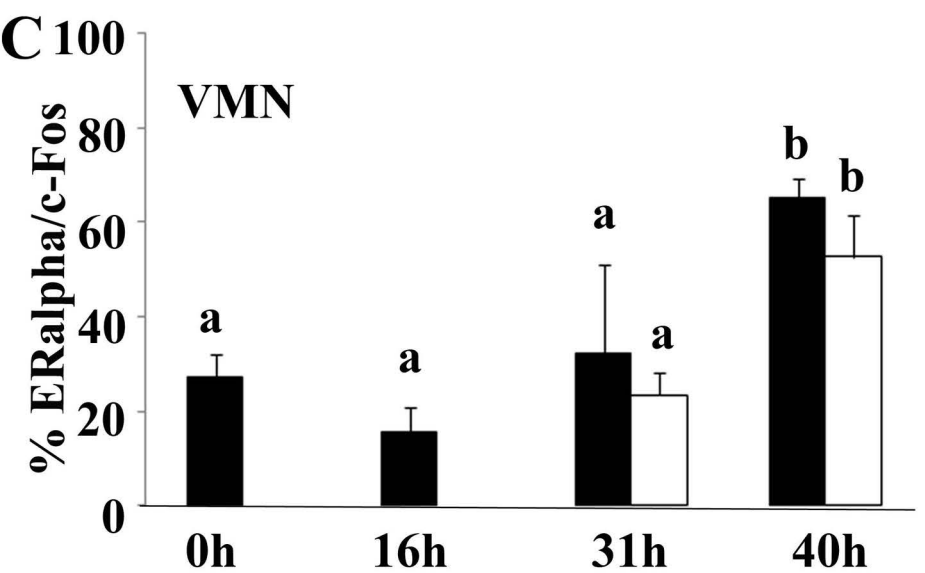
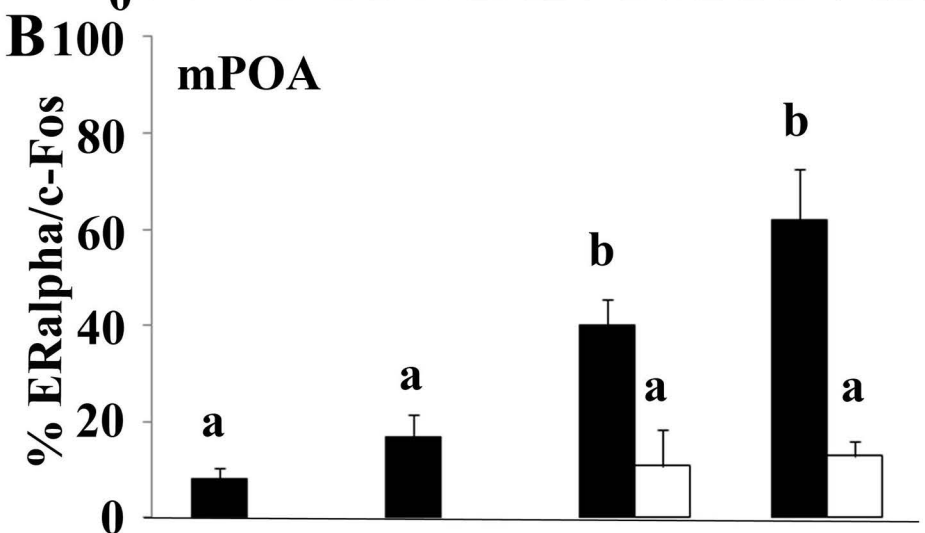
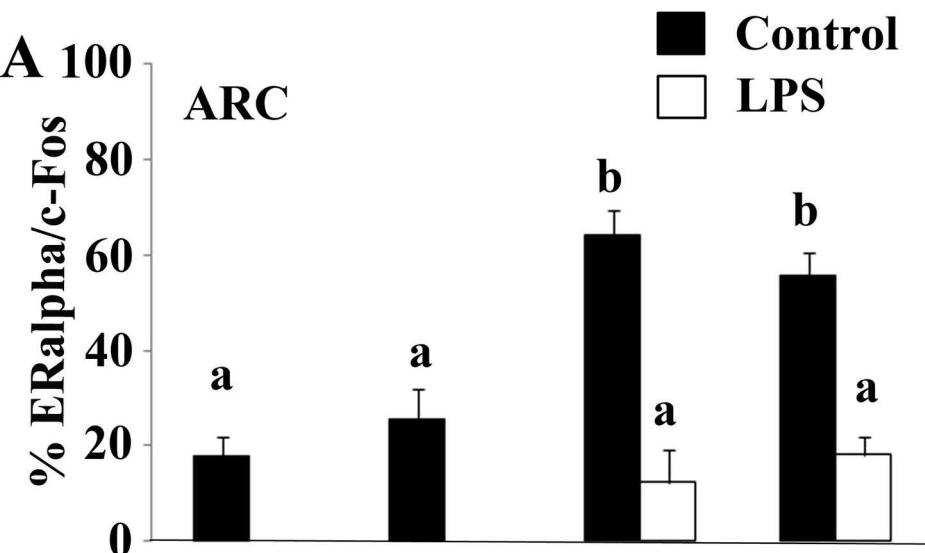
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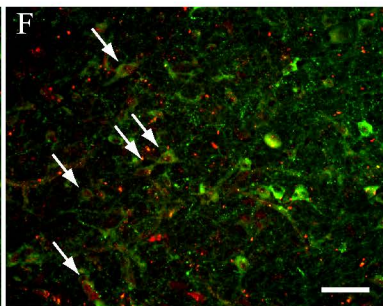
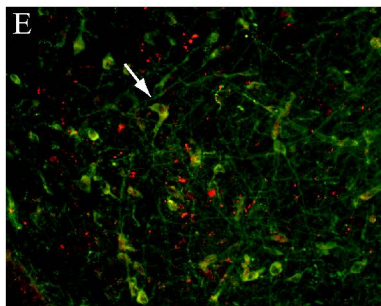
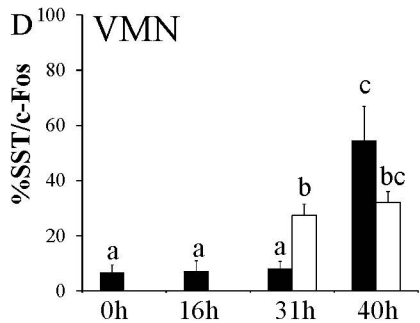
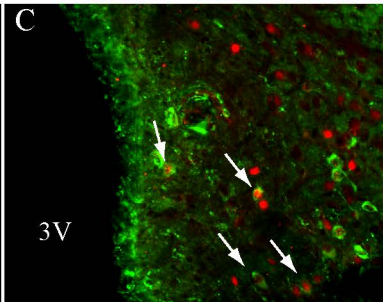
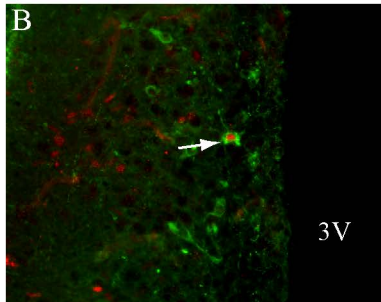
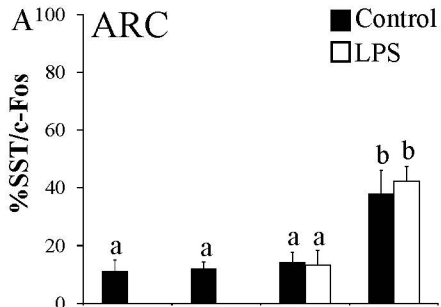












Group	Total number of ER α positive cells		
	Region		
	ARC	mPOA	VMN
0 h	52.3 \pm 26.9	15.2 \pm 4.1	38.0 \pm 8.3
16 h	57.9 \pm 19.5	17.2 \pm 3.4	49.8 \pm 16.4
31 h control	96.6 \pm 21.1	39.5 \pm 13.0	49.6 \pm 14.4
31h LPS	41.3 \pm 16.6	37.1 \pm 22.5	78.0 \pm 12.9
40h control	89.7 \pm 19.3	59.0 \pm 26.2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