

1 **KNDy neurone activation prior to the LH surge of the ewe is disrupted by LPS**

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8 **Short title:** Activated ARC cells in the follicular phase.

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10 **Keywords:** Kisspeptin, Neurokinin B, Dynorphin, LH surge, LPS, stress, ewe.

11 ABSTRACT

12 In the ewe, steroid hormones act on the hypothalamic arcuate nucleus (ARC) to initiate the
13 GnRH/LH surge. Within the ARC, steroid signal transduction may be mediated by dopamine,
14 β -endorphin or neuropeptide Y (NPY) expressing cells, as well as those co-localising
15 kisspeptin, neurokinin B (NKB), and dynorphin (termed KNDy). We investigated the time
16 during the follicular phase when these cells become activated (i.e., co-localise c-Fos) relative
17 to the timing of the LH surge onset and may, therefore, be involved in the surge generating
18 mechanism. Furthermore, we aimed to elucidate whether these activation patterns are altered
19 after lipopolysaccharide (LPS) administration, which is known to inhibit the LH surge.
20 Follicular phases of ewes were synchronised by progesterone withdrawal and blood samples
21 collected every 2 hours. Hypothalamic tissue was retrieved at various times during the
22 follicular phase with or without administration of LPS (100ng/kg). The percentage of
23 activated dopamine cells decreased before the onset of sexual behaviour, whereas activation
24 of β -endorphin decreased and NPY activation tended to increase during the LH surge. These
25 patterns were not disturbed by LPS administration. Maximal co-expression of c-Fos in
26 dynorphin immunoreactive neurones was observed earlier during the follicular phase,
27 compared to kisspeptin and NKB, which were maximally activated during the surge. This
28 indicates a distinct role for ARC dynorphin in the LH surge generation mechanism. Acute
29 LPS decreased the percentage of activated dynorphin and kisspeptin immunoreactive cells.
30 Thus, in the ovary-intact ewe, KNDy neurones are activated prior to the LH surge onset and
31 this pattern is inhibited by the administration of LPS.

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35 INTRODUCTION

36 During the late follicular phase of the ewe, the decrease in plasma progesterone
 37 concentrations after luteolysis, along with an increase of oestradiol from the dominant
 38 follicle(s), triggers the onset of sexual behaviour, closely followed by a sudden and massive
 39 release of gonadotrophin-releasing hormone (GnRH) and therefore, luteinising hormone (LH),
 40 leading to ovulation. By contrast, various types of stressors, such as an acute bolus injection of
 41 the *E. coli* endotoxin (lipopolysaccharide; LPS) during the late follicular phase decreases
 42 plasma oestradiol concentrations and abolishes both sexual behaviour and the LH surge
 43 (Fergani, et al. 2012) via mechanisms that remain largely unknown.

44 In the ewe, oestradiol acts, at least in part, in the vicinity of the arcuate nucleus (ARC) to
 45 initiate positive feedback mechanisms (Blache, et al. 1991, Caraty, et al. 1998). In line with
 46 this hypothesis, we have recently shown that the number of ER α -containing cells that are
 47 activated in the ARC (as measured by co-localisation with c-Fos) increases dramatically at least
 48 6-7 hours prior to the surge onset and remains elevated throughout the LH surge (Fergani, et
 49 al. 2014). Furthermore, this pattern of activation is attenuated, if preceded by acute
 50 administration of LPS (Fergani, et al. 2014). Undoubtedly, kisspeptin signaling is the key
 51 pathway in mediating oestradiol positive feedback on GnRH neurones in all species studied to
 52 date (Fergani, et al. 2013, Lehman, et al. 2010, Smith 2009) and is therefore a primary candidate
 53 for initiating the GnRH/LH surge. However, ARC kisspeptin cells are activated during the LH
 54 surge but not at other times in the follicular phase (Fergani, et al. 2013, Merkley, et al. 2012).
 55 Thus, there are other cell types activated in the ARC before the expected surge onset that are
 56 not kisspeptin immunoreactive cells, but contain ER α .

57 In this context, the ARC contains tyrosine hydroxylase (TH; a biosynthetic enzyme marker
 58 for dopamine), β -endorphin and neuropeptide Y (NPY) cells, sub-populations of which contain

ER α (Antonopoulos, et al. 1989, Lehman, et al. 1993, Lehman and Karsch 1993) and have been implicated in reproductive neuroendocrine mechanisms, and also in the pathophysiology of stress-induced reproductive disruptions (Fabre-Nys, et al. 2003, Melis and Argiolas 1995, Taylor, et al. 2007) These neuropeptides are, therefore, potential candidates for contributing to the generation of sexual behaviour and/or GnRH surge secretion. More importantly, however, nearly all kisspeptin cells in the ARC co-localise two other neuropeptides that are key in the control of GnRH secretion: neurokinin B (NKB) and dynorphin (Goodman, et al. 2007, Navarro, et al. 2011) and thus are termed KNDy cells (Kisspeptin, Neurokinin B and Dynorphin; (Cheng, et al. 2010, Navarro, et al. 2011). As 94% of kisspeptin cells co-localise dynorphin and 80% co-localise NKB, with an equally high reciprocal co-localisation (Goodman, et al. 2007), immunohistochemical detection of kisspeptin protein would potentially reflect presence of all three neuropeptides. However, in the ewe, KNDy peptide immunoreactivity and/or gene expression fluctuates depending on hormonal and gonadal status (Foradori, et al. 2006, Smith 2009). Thus, it is plausible to speculate that in ovary-intact ewes, endogenous fluctuation of the ovarian steroid hormone milieu during the follicular phase may be associated with differential protein expression within KNDy cells and, therefore, different activation patterns for each neuropeptide.

Lastly, there is evidence that kisspeptin neurones mediate the effects of stressors on the reproductive neuroendocrine axis. For example, there is down-regulation of the hypothalamic kisspeptin system in rats after administration of LPS (Iwasa, et al. 2008, Kinsey-Jones, et al. 2009). Furthermore, immunohistochemical analysis of kisspeptin combined with c-Fos, revealed that LPS administration is accompanied by reduced activation of kisspeptin cells in the ARC of the ewe (Fergani, et al. 2013, 2014). However, the effects of stressors on levels dynorphin and NKB immunoreactivity and activation in the ARC haven't received as much attention.

We have shown that activation of ER α -containing cells in the ARC dramatically increases at least 6-7 hours prior to the LH surge onset and this pattern is attenuated if preceded by a bolus injection of LPS (Fergani, et al. 2014). Furthermore, at least some of these ER α -containing cells are not kisspeptin cells and therefore, their phenotype remains to be elucidated (Fergani, et al. 2013, 2014). For the present study, we collected brain tissue from ewes at various times in the follicular phase and used immunohistochemistry to pinpoint the time when dopamine, β -endorphin, NPY and kisspeptin-NKB-dynorphin (KNDy) expressing cells become activated (i.e., co-express c-Fos). Furthermore, these activation patterns were correlated to peripheral plasma oestradiol and progesterone concentrations as well as the timing of different sexual behaviours and the LH surge onset. Lastly, we sought to determine whether the disruption of sexual behaviour and/or the surge after LPS administration is associated with altered activation of any or all of these cell types, adding to the evidence of their involvement in the physiological oestrus or surge generating mechanism.

MATERIALS AND METHODS

Animals, Study Design, Tissue Collection, Blood Collection, and Hormone Assays

All procedures were conducted within requirements of the UK Animal (Scientific Procedures) Act 1986, and approved by the University of Liverpool Animal Welfare Committee. Experiments were carried out on adult, ovary-intact Lley crossbred ewes (6 groups of 4-6 ewes per group) during the mid-breeding season. After follicular phase synchronisation, ewe and ram oestrus behaviour was monitored during 30-minute observation periods before each blood sample collection at 0 h, 16 h, 24 h and subsequently at 2 h intervals till 40 h after PW (PW; progesterone intravaginal device withdrawal). The following behaviours of oestrus were recorded: 1) ewe is within one metre of a ram [behavioural scan sampling; (Martin 1986)], 2) ram nosing perineal region of ewe, 3) ewe being nudged by ram without moving away, and 4) ewe mounted by ram without moving away. Frequent blood

109 sampling, as well as the administration of all substances, was facilitated by insertion of a silastic
 110 catheter (Dow Corning, Reading, UK). Duplicate blood samples were analyzed by Enzyme-
 111 Linked Immunosorbent Assays (ELISAs) for LH, pregnane metabolites (equivalent to, and
 112 hereafter referred to as, progesterone) or cortisol. LH results were expressed as ng equivalent
 113 of NIAMDD ovine LH 21 per ml plasma. Oestradiol was measured in duplicate by
 114 radioimmunoassay (RIA) using 0.5 ml plasma extracted with 3 ml diethyl ether followed by
 115 evaporation to dryness. Contemporary inter-assay and intra-assay coefficients of variation for
 116 LH, progesterone, cortisol and oestradiol were all less than 12%. The minimum detectable
 117 amounts were 0.02 ng/ml; 0.16 ng/ml, 0.8 ng/ml and 0.2 pg/ml and assay precisions (in the
 118 mid-range of the standard curve) were 0.1 ng/ml, 0.01 ng/ml, 0.2 ng/ml and 0.2 pg/ml,
 119 respectively. All samples from individual animals were measured in the same assay for each
 120 hormone. One group of ewes was killed at 0 h (0 h control group; n=5) and another group at
 121 16 h after PW (16 h control group; n=4). At 28 h, the remaining animals received 2 ml saline
 122 vehicle, or endotoxin (lipopolysaccharides from *E. coli* 055:B5, LPS, Sigma-Aldrich, UK; i.v.
 123 dose of 100 ng/kg body weight). The dose of LPS had been determined previously to evoke a
 124 robust increase in plasma cortisol followed by a delay of in the LH surge onset (Fergani, et al.
 125 2012). The timing of the treatments was chosen in order to precede all sexual behaviours and
 126 not just mounting. Two groups were killed at 31 h (31h control, n=6 and 31h LPS group, n=5)
 127 and two groups at 40 h after progesterone withdrawal (40h control, n=5 and 40h LPS group,
 128 n=5). Ewes were euthanised with pentobarbitone and perfused with: 2 litres 0.1M phosphate
 129 buffer (PB; pH 7.4) containing 25,000IU per litre of heparin and 1% sodium nitrate; then 2
 130 litres Zamboni fixative (4 % paraformaldehyde) and 7.5 % saturated picric acid in 0.1M PB,
 131 pH 7.4); followed by 500 ml of the same fixative containing 30 % sucrose. Hypothalamic
 132 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 °C until processed for immunohistochemistry.

Dual-labelled immunofluorescence for c-Fos and DA, β -endorphin, NPY, dynorphin, or NKB.

Dual-label immunofluorescence was carried out on 40 μ m sections containing ARC. All steps were followed by washes in 0.1M phosphate buffer saline, pH 7.2 (PBS) and 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, UK) and 0.25 % sodium azide (Sigma) in 0.1M 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) and a 72 h incubation at 4°C with polyclonal rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA; 1:5,000). Next, sections were incubated with donkey anti-rabbit Cy3 (711-165-152, Jackson ImmunoResearch, West Grove, PA; 1:500) for 2 h. A second immunofluorescent procedure was then performed, as described above, to localise the second primary antibodies: mouse anti-tyrosine hydroxylase serum (MAB318, Millipore, Billerica, MA; 1:20,000), or rabbit-anti- β -endorphin serum (T-4041, Peninsula Laboratories, San Carlos, CA; 1:500), or rabbit-anti-neuropeptide Y serum (N9528, Sigma-Aldrich, UK; 1:5,000), or rabbit-anti-dynorphin serum (T-4268, Peninsula Laboratories, LLC, San Carlos, CA; 1:10,000) or rabbit-anti-NKB serum (T-4450, Peninsula Laboratories, LLC, San Carlos, CA; 1:1,000); each incubated for 72 h at 4 °C and then visualised using donkey-anti-rabbit Dylight 488 (715-485-152, Jackson ImmunoResearch West Grove, PA; 1:500) or donkey-anti-mouse Dylight 488 (715-485-151, Jackson ImmunoResearch West Grove, PA; 1:500), accordingly. Thereafter, sections were mounted on chrome alum gelatin-coated slides and cover-slipped with Vectashield anti-fading mounting medium (H-1000, Vector Laboratories Ltd, UK). The c-Fos (Ghuman, et al. 2011), tyrosine hydroxylase (Robinson, et al. 2010), β -endorphin (Ghuman, et al. 2011), neuropeptide Y (Skinner and

Herbison 1997), kisspeptin (Franceschini, et al. 2006), dynorphin (Foradori, et al. 2006) and NKB (Goodman, et al. 2007) antibodies have been validated previously for use in ovine neural tissue.

Triple-labelled immunohistochemistry for c-Fos, kisspeptin and dynorphin

Interestingly, we observed a different dynorphin cell activation pattern compared to what we had previously reported for kisspeptin (Fergani, et al. 2013). In order to confirm this discrepancy we performed triple-label immunohistochemistry for c-fos, kisspeptin and dynorphin. This consisted of an immunoperoxidase protocol in which nuclear c-Fos was detected first with diaminobenzidine as chromogen (DAB; brown reaction product) followed by visualisation of kisspeptin and dynorphin with immunofluorescence. As kisspeptin and dynorphin antibodies were both derived in the rabbit, we used a previously described modified protocol (Cheng, et al. 2010, Hunyady, et al. 1996). Free-floating sections were washed thoroughly in PBS for 2 h to remove the cryoprotectant solution followed by a 15 min incubation in 40 % methanol and 1 % hydrogen peroxide (H₂O₂; 316989, Sigma-Aldrich, UK) in PBS to inactivate endogenous peroxidases. Sections were then incubated for 1 h in blocking solution (10 % donkey serum in PBS), followed by a 72 h incubation in rabbit anti-c-Fos antibody (1:5,000) at 4 °C. After, sections were labelled with biotinylated donkey anti-rabbit IgG (711-065-152, Jackson ImmunoResearch West Grove, PA; 1:500) for 2 h, followed by 90 min in Vectastain Elite ABC kit (1:250 in PBS; PK6100, Vector Laboratories Ltd, UK). Nuclear c-Fos was visualised by 5 min incubation in DAB (SK-4100, Vector Laboratories, Ltd, UK). The second immunohistochemical procedure consisted of incubation for 72 h in rabbit anti-kisspeptin (lot #564; gift from Prof. Alain Caraty, INRA Nouzilly, France; 1:150,000) at 4 °C. Following incubation, sections were labelled with biotinylated donkey anti-rabbit IgG (1:500; for 2 h) and then incubated in Vectastain Elite ABC kit (1:250; for 90 min). The signal was amplified in TSA for 10 min (1:200; New England Nuclear Life Science Products Life

183 Sciences, Boston, MA) diluted in PBS with 0.003 % H₂O₂ as substrate (Cheng, et al. 2010) and
184 then labelled with streptavidin conjugated AlexaFluor 488 (S11223, Molecular Probes, Eugene,
185 OR, USA; 1:100) for 2 h. A third immunohistochemical procedure was then performed with a
186 72 h incubation with rabbit anti-Dynorphin (T-4268, Peninsula Laboratories, LLC, San Carlos,
187 CA; 1:10,000) and subsequent labelling with donkey anti-rabbit Cy3 (711-165-152, Jackson
188 Immunoresearch West Grove, PA; 1:500) for 2 h. Finally, sections were washed, mounted on
189 chrome alum gelatin-coated slides, dried, and cover-slipped with Vectashield anti-fading
190 mounting medium.

191 *Data collection and analysis*

192 Hormone and immunohistochemistry data were analysed with Minitab® 15 statistical
193 package (MINITAB Inc, Pennsylvania, USA). Statistical significance was accepted when $P <$
194 0.05.

195 Quantitative analysis was carried out on three sections from each of the middle and caudal
196 divisions of the ARC from each animal, where the largest numbers of cells are located
197 (Lehman, et al. 2010). Sections were examined under an epi-fluorescent/brightfield microscope
198 (Zeiss Axio Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA
199 I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20× objective.
200 Photographs (three per section) were acquired with an image analysis program AxioVision
201 (Zeiss Imaging Systems) and consisted of single c-Fos staining, single dopamine, β -endorphin,
202 neuropeptide Y, kisspeptin, dynorphin or NKB staining as well as merged fluorescent images
203 to produce a spectral combination of green (fluorescein) and red (rhodamine). All photographs
204 were imported into Image J version 1.42q, and counts performed using the cell count plug-in.
205 Triple co-localisation was determined by switching through the single-labelled
206 brightfield/fluorescent photographs. The observer was unaware of the animal identity and
207 group. The mean total number and percentage of single-, dual- or triple-labelled cells was

summed from the photographs of each section and then averaged for each ewe and compared using GLM ANOVA, followed, when appropriate, by Tukey's multiple comparisons post hoc test. Mean data (\pm SEM), as presented in figures and results, were calculated by averaging values for each group.

The data were analyzed in two ways: the first consisted of data derived from control ewes, grouped according to time as well as hormonal and sexual behaviour status; i.e., those killed at 0 or 16 h after PW, those killed at 31 h after PW but before the onset of sexual behaviour (Before sexual behaviour, $n=3$), those killed at 31 or 40 h after PW but after the onset of sexual behaviour and before exhibiting an LH surge (During sexual behaviour, $n=5$) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, $n=3$). This grouping was used to describe the timing of each neuropeptide cell activation relative to the LH surge onset. Secondly, control and treated ewes were grouped according to time of killing after PW, and these data were used to compare LPS treatment effects.

Lastly, regression analysis was used to correlate plasma oestradiol and progesterone concentrations (percentage of change from 0 h) to the percentage of DA, β -endorphin, NPY, kisspeptin, dynorphin or NKB cells that co-localised c-Fos (i.e., were activated) during various times in the follicular phase of control ewes.

RESULTS

Animals treated with LPS did not show any signs of illness, with very few exceptions of mild coughing and increased respiration rate.

Luteinising hormone (LH), sexual behaviour and plasma hormone profiles

Detailed LH, sexual behaviour and plasma hormone profiles have been published previously (Fergani, et al. 2013). In brief, there was no sexual behaviour or LH surge recorded in control ewes killed at 0 or 16 h. Eight of eleven control animals, killed at 31 or 40 h, began exhibiting sexual behaviour at 28.5 ± 2.4 h after PW, and three of five (from the 40 h control group)

233 additionally had an LH surge with a mean onset at 36.7 ± 1.3 h after PW. None of the LPS
 234 treated ewes exhibited an LH surge within the 40 h of study and only 3 out of 8 LPS treated
 235 ewes displayed early signs of oestrus behavior (near ram and being nosed) which ceased after
 236 treatment (Fergani, et al. 2013).

237 In control ewes, plasma oestradiol concentrations began to increase at 28 h after PW and
 238 reached maximum values just before the LH surge onset (i.e., at 32 h after PW; 12.2 ± 1.8
 239 pg/ml). However, treatment with LPS was followed by a decrease in oestradiol concentrations,
 240 which was evident 8 h after LPS administration (from 11.6 ± 1.6 pg/ml to 6.9 ± 1.8 pg/ml) and
 241 remained low until ewes were killed at 40 h. Plasma progesterone concentrations decreased
 242 from 0 to 28 h after PW in all groups (from 33.7 ± 2.0 ng/ml to 6.6 ± 0.4 ng/ml). However, LPS
 243 treatment increased progesterone concentrations from 6.9 ± 1.0 ng/ml before treatment to a
 244 mean maximum of 9.9 ± 1.6 ng/ml after treatment. In all control animals, mean plasma cortisol
 245 concentrations remained low throughout the study (10.5 ± 0.7 ng/ml). By contrast, LPS
 246 treatment increased cortisol concentrations to a mean maximum of 157 ± 19.8 ng/ml 2 h after
 247 treatment (Fergani, et al. 2012).

248 ***Control Ewes Grouped According to Sexual Behaviour and the LH Surge.***

249 *c-Fos co-expression with dopamine or β -endorphin or NPY:* The percentage of activated
 250 dopamine cells (% dopamine/c-Fos) decreased in the 'Before sexual behaviour' group till the
 251 'Surge' ($P < 0.05$ for each comparison; Fig. 1A), whereas the % β -endorphin/c-Fos cells was
 252 greater in the 16 h and 'During sexual behaviour' groups compared to 0 h and 'Surge' ($P < 0.05$
 253 for both comparisons; Fig. 1B). In contrast, the % NPY/c-Fos cells did not fluctuate, but tended
 254 to be higher in the 'Surge' group ($P < 0.08$; Fig. 1C). The % dopamine/c-Fos cells in the ARC
 255 was positively associated with progesterone concentrations ($P = 0.001$; Fig. 1D), whereas, %
 256 NPY/c-Fos was positively associated with plasma oestradiol concentrations ($P = 0.008$; Fig.
 257 1F).

258 *c-Fos co-expression with kisspeptin, dynorphin or NKB:*

259 The double-label immunofluorescence study (c-Fos and dynorphin) showed that the %
 260 dynorphin/c-Fos was greatest in the 'Before behaviour' group compared to earlier stages in the
 261 follicular phase ($P < 0.05$; Fig. 2). Thereafter, there was a gradual decrease until the LH surge
 262 (Fig. 2). This was a surprising result, as we have previously shown that the greatest number of
 263 kisspeptin cells express c-Fos during the surge (Fergani, et al. 2013). Therefore, we proceeded
 264 with co-staining hypothalamic sections with both proteins (kisspeptin and dynorphin) in
 265 addition to c-Fos, to confirm this novel finding.

266 The % kisspeptin/c-Fos sequentially increased during the follicular phase, with a two-fold
 267 increase during the 'Surge' ($P < 0.05$; Fig. 3A). By contrast, a two-fold increase in the %
 268 dynorphin/c-Fos was observed earlier, in the 'Before sexual behaviour' group, compared to 0
 269 and 16 h ($P < 0.05$ for both; Fig. 3B). This increase was maintained to a lesser extent till the
 270 'Surge' (Fig. 3B). The % NKB/c-Fos followed a similar pattern to that of kisspeptin and
 271 sequentially increased from 0 h till the 'Surge' ($P < 0.05$ for 'Surge' compared to other stages;
 272 Fig. 3C). Furthermore, the % kisspeptin/c-Fos and % NKB/c-Fos were positively associated
 273 with changes in oestradiol concentration ($P = 0.005$, RSq = 36 % and $P = 0.002$, RSq = 41 %
 274 for kisspeptin and NKB, respectively; Fig 3D, 3E), whereas the activation of all three
 275 neuropeptides was negatively associated with plasma progesterone values ($P = 0.002$, RSq =
 276 41 %, $P = 0.001$ RSq = 47 % and $P = 0.001$, RSq = 50 % for kisspeptin, dynorphin and NKB,
 277 respectively; Fig. 3D, 3E, 3F).

278 *Kisspeptin, dynorphin and NKB cell numbers and percentage co-localisation:* In order to
 279 address the differential activation of kisspeptin and dynorphin neurones we analyzed the
 280 colocalisation between these two peptides throughout the follicular phase. Kisspeptin and
 281 dynorphin cell numbers were greater 'During sexual behaviour' ($P < 0.05$; Fig. 4A, 4B)
 282 whereas, the number of NKB cells was greater during the 'Surge' as well ($P < 0.05$ for all

comparisons; Fig. 4C). However, the % co-localisation between kisspeptin and dynorphin immunoreactivity in the ARC varied during the follicular phase (Fig. 4D, 4E). The % of kisspeptin cells co-localising dynorphin was lower in the ‘Surge’ group [that is, there were more single-labelled kisspeptin cells ($P < 0.05$ for all comparisons; Fig. 4D)]. Furthermore, there were fewer dynorphin cells co-localising kisspeptin in the ‘Before sexual behaviour’ group (that is, there were more single-labelled dynorphin cells at those times; $P < 0.05$ for all comparisons; Fig. 4A-4D).

Finally, the *total number* of activated dynorphin cells was greater in the ‘Before sexual behaviour’ group compared to the activated kisspeptin cells ($P < 0.05$; Fig. 4F), whereas there were more kisspeptin activated cells during the ‘Surge’ compared to activated dynorphin cells ($P < 0.05$; Fig. 4F). Examples of photomicrographs of sections from the ARC that have been triple-labelled for c-Fos, kisspeptin and dynorphin as well as c-Fos and NKB are shown in Fig 5.

Comparison of control and LPS treated ewes

To determine the effects of LPS treatment, data was analysed according to time of killing after PW (irrespective of sexual behaviour and the LH surge), and compared to control animals at each time point.

Dopamine or β -endorphin or NPY cell numbers and co-expression with c-Fos: The total number of immunoreactive dopamine cells increased in the 40 h controls compared to 0 h ($P < 0.05$; Table 1), whereas, there were more β -endorphin cells in the 31 and 40 h control and LPS groups (compared to 0 h controls $P < 0.05$ for all; Table 1). The number of NPY cells did not vary across the follicular phase of controls and was not affected by treatment (Table 1). The % dopamine/c-Fos, % β -endorphin/c-Fos and % NPY/c-Fos was not affected by LPS treatment (Fig. 6A, 6B, 6C).

Kisspeptin, dynorphin and NKB cell numbers and co-expression with c-Fos: In controls, maximum kisspeptin and dynorphin cell numbers were recorded at 31 and 40 h after PW (Table 2). Of note, there were more dynorphin cells than kisspeptin or NKB cells in the 31 h controls (Table 2). Treatment with LPS decreased the number of immunodetectable dynorphin cells compared to controls (Table 2). The number of NKB cells did not vary during the follicular phase, or after LPS treatment (Table 2).

At 40 h after PW (i.e., 12 h after LPS administration), the % kisspeptin/c-Fos was markedly lower in LPS treated animals compared to controls ($P < 0.05$; Fig. 6D). Interestingly, the % dynorphin/c-Fos decreased earlier than kisspeptin, i.e., at 31 and 40 h (3 and 12 h after LPS administration; $P < 0.05$; Fig. 6E). The % NKB/c-Fos was not affected by LPS administration within the 12 hours post treatment (Fig. 6F).

DISCUSSION

The present study demonstrates that various cell types within the ARC of the ovary-intact ewe are activated at different times during the follicular phase, leading up to the GnRH/LH surge. Specifically, activation of dopamine neurones was initially high, but decreased before the onset of sexual behavior; whereas the activation of β -endorphin cells increased in the mid-follicular phase, decreasing a few hours later during the surge. The percentage of activated NPY cells tended to increase in animals undergoing an LH surge. Treatment with LPS had no effect on the activation of dopamine, β -endorphin or NPY cells raising the possibility that these cell types are only permissive in the surge induction process. Our observations are also consistent with a role for KNDy cells in the GnRH/LH surge mechanism as these cells became activated prior to the LH surge onset. Interestingly, in our ovary-intact ewe model, kisspeptin, NKB and dynorphin immunoreactivity and co-localisation vary throughout the follicular phase, leading to differential activation patterns for each individual KNDy peptide. Maximum kisspeptin and NKB immunoreactive cells were maximally activated during the GnRH/LH

surge; whereas maximum activation of dynorphin positive cells occurred at least 6-7 h before that. Furthermore, LPS administration in the late follicular phase prevented kisspeptin and dynorphin positive cell activation and this was accompanied by a failure to exhibit an LH surge.

Dopamine has been implicated in the control of female sexual behavior (Fabre-Nys and Gelez 2007). In the present study, dopamine neurones in the ARC were maximally activated in the early follicular phase but greatly decreased just before the ewes began exhibiting signs of estrous. These results are consistent with a biphasic role of dopamine as described by Fabre-Nys (Fabre-Nys, et al. 1994, Fabre-Nys, et al. 2003), who showed that extra-cellular concentrations of dopamine in the mediobasal hypothalamus (MBH; containing the ARC and ventromedial nucleus; VMN) are initially high, followed by a sharp decrease preceding the onset of sexual behaviour (Fabre-Nys, et al. 1994). The present data indicate that the source of dopaminergic input in the MBH could be derived, at least in part, from cells located in the ARC. Indeed, 20% of dopamine neurones in the ARC send projections towards the VMN (Qi, et al. 2008) providing a possible signaling pathway involved in the initiation of sexual behaviours. However, in the present study, ewes treated with LPS did not exhibit signs of sexual behaviour but dopamine cell activation in the ARC was not affected, indicating that this pathway may be permissive but not indispensable for the initiation of oestrus.

In the ARC, 15-20 % of β -endorphin cells contain ER α (Lehman and Karsch 1993), and β -endorphin or pro-opiomelanocortin (POMC) fibres directly innervate GnRH cells in the rat (Leranth, et al. 1988) and monkey (Thind and Goldsmith 1988) or form close appositions in the ewe (Dufourny, et al. 2005). In the present study, activation of β -endorphin cells slightly increased during the mid/late follicular phase, but not in animals exhibiting a GnRH/LH surge. These results are consistent with those of Domanski (Domanski, et al. 1991), who demonstrated a decrease in β -endorphin concentrations in the ARC of ovary-intact ewes before the onset of the pre-ovulatory LH surge, but conflict with those of Taylor *et al.*, (Taylor, et al.

2007) who observed an increase in POMC mRNA at the time of the peak of the GnRH surge in OVX ewes. The reason for this divergence between studies is not known, although it may reflect differences in the timing of brain tissue sampling, as well methods of detection (i.e., protein *versus* gene expression). Furthermore, various POMC gene products other than β -endorphin, such as α -melanocyte stimulating hormone may have differential effects on the reproductive axis compared to β -endorphin (Gonzalez, et al. 1997, Scimonelli, et al. 2000).

In the sheep, the role of NPY in the regulation of GnRH is not clear. NPY administered intracerebroventricularly (icv) suppressed release of LH in OVX and OVX oestradiol-treated sheep (Estrada, et al. 2003, Malven, et al. 1992), whereas in follicular phase ewes, icv administration of anti-NPY serum delayed the onset of the pre-ovulatory GnRH/LH surge, implying a stimulatory role in this process (Porter, et al. 1993). In addition, a stimulatory effect on GnRH release by NPY infusion into the ME was observed in ovary-intact ewes, but only in the follicular, and not in the luteal, phase (Advis, et al. 2003). In the present study, NPY activation tended to be higher in animals that were exhibiting an LH surge. Furthermore, this pattern of activation was positively correlated to plasma oestradiol concentrations. It is plausible to speculate that NPY is involved in the regulation of GnRH secretion (Kalra, et al. 1991, Sahu, et al. 1995) but specific actions depend on the prevailing endocrine status. For example, in rats NPY stimulates GnRH release in the presence of oestradiol, but inhibits GnRH release during absence of sex steroids (Kalra and Crowley 1992). Interestingly, LPS had no effect on β -endorphin or NPY cell activation. Therefore, it appears that neither of these phenotypes are essential in the surge induction process, nor to mediate the LPS-induced disruption of sexual behaviour or the GnRH/LH surge.

The immunoreactivity of all three KNDy peptides in the ARC was greater in the late, rather than early, follicular phase, adding to the increased evidence for the involvement of these cells in oestradiol positive feedback in the ewe. We have shown that kisspeptin cells are activated

382 during the LH surge in ovary-intact ewes (Fergani, et al. 2013) and a similar finding has been
 383 reported by Merkley *et al.*, (Merkley, et al. 2012) in OVX oestrogen-treated sheep undergoing
 384 an LH surge. More recently, an important role for NKB in oestradiol positive feedback and the
 385 GnRH surge has emerged as local administration of an NKB receptor agonist (senktide) into
 386 the retrochiasmatic area stimulates surge-like LH secretion (Billings, et al. 2010) whereas an
 387 NKB receptor antagonist (SB222200) administered in the same region decreased LH surge
 388 amplitude (Porter, et al. 2014). NKB neurones located in the ARC are thought to be the source
 389 of input to this area (Grachev, et al. 2016). In the present study, NKB cells in the ARC were
 390 gradually activated, with maximum activation during the LH surge (i.e., in a similar pattern to
 391 kisspeptin cells). Furthermore, there was a positive correlation of kisspeptin and NKB cell
 392 activation with plasma oestradiol concentrations and a negative correlation with progesterone.
 393 These data provide further evidence that kisspeptin and NKB neurones in the ARC are activated
 394 during, and may therefore be involved in, oestradiol positive feedback and the surge phase of
 395 GnRH/LH secretion in the ewe.

396 To date, dynorphin neurones in the ARC of the ewe have been implicated in the negative
 397 feedback actions of progesterone to inhibit GnRH and LH pulse frequency (Goodman, et al.
 398 2011). Interestingly, we observed maximum activation of dynorphin immunoreactive cells
 399 occurred at least 6-7 h before the expected LH surge, at a time when activation of kisspeptin
 400 and NKB were comparatively lower, suggesting that dynorphin may play a distinct role in the
 401 GnRH surge induction process. The precise physiological role of an increase in dynorphin
 402 protein within KNDy cells prior to the LH surge is not known, however, these observations are
 403 consistent with the hypothesis that endogenous opioid systems in the hypothalamus are
 404 permissive of sexual behaviour and the GnRH/LH surge (Kalra 1993, Walsh and Clarke 1996)
 405 and may, therefore, be a critical part of the oestradiol positive feedback mechanism (Smith and
 406 Gallo 1997, Zhang and Gallo 2003). Furthermore, recent evidence suggests that this dynorphin

407 input originates from ARC KNDy cells, as ablation of these cells leads to an abnormal increase
408 in the amplitude of the LH surge, whereas microinjections of dynorphin in to the POA
409 of KNDy-ablated rats restored LH surge levels (Helena, et al. 2015). We speculate that
410 increased opioid influence during the mid-follicular phase plays a role in preventing premature
411 activation of GnRH neurones, giving time for an increase in the releasable pool of GnRH, as
412 well as an increase in GnRH receptor numbers in the pituitary (Clarke, et al. 1988, Walsh and
413 Clarke 1996).

414 Different activation patterns between kisspeptin/NKB and dynorphin is a novel finding in
415 the present study, as all three neuropeptides co-localize in the same KNDy cell, as has been
416 described in OVX ewes (Goodman, et al. 2007). However, this can be explained by considering
417 the expression of individual KNDy peptides as being differentially regulated by steroid
418 hormones and gonadal status. For example, ovariectomy increases NKB and kisspeptin, but
419 decreases dynorphin gene and protein expression in the sheep ARC (Foradori, et al. 2005,
420 Navarro, et al. 2009, Pilon, et al. 2003). Furthermore, recent findings in the rat, using
421 immunoelectron microscopy, indicate that each neuropeptide is contained within separate
422 neurosecretory vesicles, adding to the evidence that each KNDy peptide is differentially
423 regulated within the KNDy neurone (Murakawa, et al. 2016). Therefore, we hypothesise that
424 fluctuating endogenous steroid concentrations in ovary-intact ewes result in differential peptide
425 content and/or immunoreactivity within the KNDy neurone, leading to increased dynorphin
426 immunoreactivity (and subsequently, activation), 6-7 hours before the LH surge.

427 In the present study, acute LPS administration in the late follicular phase was accompanied
428 by suppression of dynorphin and kisspeptin activation within the KNDy cell, a decrease in
429 plasma oestradiol concentrations, and subsequent absence of a GnRH/LH surge. Several
430 studies report down-regulation of the ARC kisspeptin system in rats and male rhesus monkeys
431 after metabolic or immune/inflammatory stressors, such as negative energy balance

(Castellano, et al. 2005), short term fasting (Wahab, et al. 2010) or administration of LPS (Iwasa, et al. 2008, Kinsey-Jones, et al. 2009). However, to the best of our knowledge, there are no equivalent data for the actions of stressors on dynorphin cells located in the ARC. Regarding the potential mechanisms via which LPS inhibited the activation of kisspeptin and dynorphin within KNDy cells, it must be noted that plasma oestradiol concentrations decreased 8 h after administration of LPS (Fergani, et al. 2013), presumably via inhibition of GnRH/LH pulses and deprivation of mature follicle(s) gonadotrophic drive. However, the decrease in the percentage of activated dynorphin neurones occurred sooner (within 3 h after LPS treatment) and therefore, lack of an efficient oestradiol signal cannot be the cause but could be the result of lack in KNDy cell activation. Various other factors have been implicated in LPS-induced disruption of the oestrous cycle and at least some of those may be acting upon KNDy neurons. In our paradigm of an acute peripheral LPS administration, peripheral cortisol and progesterone concentrations increased within 2 h after the injection and are, therefore, potential candidates for the immediate inhibition of dynorphin neurone activity and the surge mechanism. In accordance, Pierce et al., (Pierce, et al. 2008) and Wagenmaker et al., (Wagenmaker, et al. 2009) report that cortisol disrupts the positive feedback effect of oestradiol to trigger an LH surge in the ewe, whereas progesterone has been implicated in both inhibition of GnRH pulses (Karsch, et al. 1987) and of the surge mechanism (Kasa-Vubu, et al. 1992, Richter, et al. 2005). Glucocorticoid receptors (GR) co-localize with kisspeptin neurons in mice and rats (Takumi, et al. 2012) and the tissue specific deletion of GR in kisspeptin neurons eliminates cortisol-induced suppression of kisspeptin gene expression (Grachev, et al. 2013). However, the absence of GR in kisspeptin neurones does not prevent the suppression of the reproductive axis following traumatic stress (Whirledge and Cidlowski 2013), and thus, GR signaling in KNDy neurons cannot fully account for LH surge disruption. Similarly, we have previously presented evidence that the

progesterone/glucocorticoid receptor antagonist, RU486, was unable to reverse delays in the GnRH/LH surge induced by a metabolic stressor (Dobson and Smith 2000). Notably, corticotrophin releasing hormone (CRH) has been demonstrated to be a powerful suppressor of the GnRH pulse generator in the rat (Li, et al. 2010), whereas acute LPS administration increased the number of immunoreactive cells within the ARC/ME that contained CRH-type 2 receptors (Fergani, et al. 2013). However, icv administration of CRF in the sheep either increases (Naylor, et al. 1990, Caraty, et al. 1997) or has no effect (Clarke, et al. 1990) on LH pulse frequency. Clearly, this pathway requires further investigation.

Lastly, the action of interleukin- (IL-)1 β must also be taken in to account, as this cytokine is secreted in response to LPS and is considered to be the most potent down regulator of reproductive processes during an immune/inflammatory challenge (Herman, et al. 2012). IL-1 β has been described to act within the hypothalamus by inhibiting GnRH expression but also directly on pituitary gonadotropes to suppress GnRH receptor expression (Herman, et al. 2013, Herman, et al. 2012). Whether IL-1 β has any direct or indirect inhibitory effect on ARC KNDy neurones, merits further investigation.

Our results indicate that the activation patterns of ARC cells containing dopamine, β -endorphin, and NPY differs throughout the follicular phase of ovary-intact ewes. However, a surge-inhibiting dose of LPS had no effect on the activation of these phenotypes, suggesting that they are not essential mediators of GnRH/LH surge release. More importantly, our results confirm a critical role for KNDy cells in the GnRH/LH surge mechanism in the ewe. Furthermore, cells immunoreactive for dynorphin were activated at least 6-7 h before the expected LH surge, at a time when activation of kisspeptin and NKB positive cells was comparatively lower, suggesting that dynorphin, possibly derived from KNDy cells, may play a distinct role in the GnRH surge induction process. The physiological relevance of this finding remains to be explored.

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483 There is no conflict of interest that could be perceived as prejudicing the impartiality of the
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485

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1 **Figure Legends**

2 **FIG. 1. A, B and C:** Mean % (\pm SEM) dopamine, β -endorphin and neuropeptide Y cells,
 3 respectively, that co-express c-Fos in the ARC at different stages during the follicular phase of
 4 control ewes as determined by dual-immunofluorescence. Animals were grouped according to
 5 time after PW as well as by hormonal and behavioural status, that is, grouped into those killed
 6 at 0 and 16 h after PW ($n = 4-5$), those killed before the onset of sexual behaviour (before
 7 sexual behaviour, $n = 3$), those killed after the onset of sexual behaviour but before exhibiting
 8 an LH surge (during sexual behaviour, $n = 5$), and those killed during sexual behaviour and an
 9 LH surge(surge, $n = 3$). Within each panel, differences between the percentages are indicated
 10 by different letters on top of each bar ($P < 0.05$) except * when $P < 0.08$. **D, E and F:**
 11 Regression graphs showing the association between dopamine, β -endorphin and neuropeptide
 12 Y cells, respectively, that co-express c-Fos in the ARC of control ewes against the % change in
 13 concentration from 0 h to the mean two consecutive highest or lowest concentrations for
 14 oestradiol (o, E; dashed line) or progesterone (■, P; solid line), respectively.

15 **FIG. 2.** Mean % (\pm SEM) dynorphin cells that co-localise c-Fos in the ARC at various stages
 16 during the follicular phase of control ewes as determined by dual-labell immunohistochemistry
 17 (Dynorphin/c-Fos). Animals are grouped according to time as well as hormonal and
 18 behavioural status (for details, see Fig. 1 legend). Within each panel, differences between the
 19 percentages are indicated by different letters on top of each bar ($P < 0.05$).

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23 **FIG. 3. Panels A, B, C:** Mean % (\pm SEM) kisspeptin, dynorphin and NKB cells, respectively,
 24 that co-localise c-Fos in the ARC at various stages during the follicular phase of control ewes
 25 as determined by triple- and dual-labell immunohistochemistry (kisspeptin/dynorphin/c-Fos

26 and NKB/c-Fos, respectively). Animals are grouped according to time as well as hormonal and
 27 behavioural status (for details, see Fig. 1 legend). Within each panel, differences between the
 28 percentages are indicated by different letters on top of each bar ($P < 0.05$). **D**, **E** and **F**:
 29 Regression graphs showing the association between the % kisspeptin, % dynorphin and %
 30 NKB cells, respectively, that co-localise c-Fos in the ARC against the % change in
 31 concentration from 0h to the mean two consecutive highest or lowest concentrations for
 32 oestradiol (o, E; dashed line) or progesterone (■, P; solid line), respectively.

33 **FIG. 4. A, B and C:** Mean (\pm SEM) number of kisspeptin, dynorphin and NKB cells,
 34 respectively. **D:** mean % (\pm SEM) kisspeptin cells co-localising dynorphin, and **E:** mean %
 35 (\pm SEM) dynorphin cells co-localising kisspeptin. **F:** mean (\pm SEM) number of dynorphin (black
 36 bars) and kisspeptin (white bars) cells that co-localise c-Fos. Mean (\pm SEM) numbers and
 37 percentages are per section from the ARC at various stages during the follicular phase of control
 38 ewes as determined by triple- and dual-labell immunohistochemistry (kisspeptin/dynorphin/c-
 39 Fos and NKB/c-Fos, respectively). Animals are grouped according to time as well as hormonal
 40 and behavioural status (for details, see Fig. 1 legend). Within each panel and type of cell,
 41 differences between numbers and percentages are indicated by different letters on top of each
 42 bar ($P < 0.05$). **F:** * $P < 0.05$ compared to activated dynorphin cells.

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44 **FIG. 5. A, B and C:** Sets of photomicrographs in the ARC that were triple-labelled for c-
 45 Fos, kisspeptin and dynorphin, as well as a merged image (**D**) in control animals at 31 h after
 46 progesterone withdrawal (that is, before the onset of sexual behavior or the LH surge).
 47 Photomicrographs from the ARC nucleus that were dual-labelled for NKB cells and their co-
 48 localisation with c-Fos in control before the LH surge, but during sexual behaviour (**E**) as well
 49 as in control animals at 40 h and specifically during an LH surge (**F**). **A-D:** Arrows indicate

50 examples of single-labelled dynorphin cells co-localising c-Fos. **E-F**: Arrows indicate
 51 examples of dual-labelled cells. Original magnification: $\times 20$ (A-E), original magnification: $\times 10$
 52 (F). 3V = third ventricle.

53 **FIG. 6. A, B, C, D, E, F:** Mean % (\pm SEM) dopamine, β -endorphin, neuropeptide Y,
 54 kisspeptin, dynorphin and NKB cells, respectively, that co-express c-Fos in the ARC across the
 55 follicular phase of control ewes as determined by immunofluorescence. Animals are grouped
 56 according to killing time after progesterone withdrawal (PW), that is, control ewes at 0, 16, 31
 57 and 40h (n=4-6 per group; black bars) as well as after LPS at 31 and 40h (n=4 for both times;
 58 white bars). Treatment with LPS was at 28h after PW. Within each panel, differences within
 59 controls are indicated by different letters on top of each bar ($P < 0.05$). Differences between
 60 control and LPS treated ewes, at each time point, are indicated with a star ($P < 0.05$).

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