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

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ABSTRACT

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

INTRODUCTION

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

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

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

ERα (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 ERα-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 ERα-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 where 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 Lleyn 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

sampling, as well as the administration of all substances, was facilitated by insertion of a silastic catheter (Dow Corning, Reading, UK). Duplicate blood samples were analyzed by Enzyme-Linked Immunosorbent Assays (ELISAs) for LH, pregnane metabolites (equivalent to, and hereafter referred to as, progesterone) or cortisol. LH results were expressed as ng equivalent of NIAMDD ovine LH 21 per ml plasma. Oestradiol was measured in duplicate by radioimmunoassay (RIA) using 0.5 ml plasma extracted with 3 ml diethyl ether followed by evaporation to dryness. Contemporary inter-assay and intra-assay coefficients of variation for LH, progesterone, cortisol and oestradiol were all less than 12%. The minimum detectable amounts were 0.02 ng/ml; 0.16 ng/ml, 0.8 ng/ml and 0.2 pg/ml and assay precisions (in the mid-range of the standard curve) were 0.1 ng/ml, 0.01 ng/ml, 0.2 ng/ml and 0.2 pg/ml, respectively. All samples from individual animals were measured in the same assay for each hormone. One group of ewes was killed at 0 h (0 h control group; n=5) and another group at 16 h after PW (16 h control group; n=4). At 28 h, the remaining animals received 2 ml saline vehicle, or endotoxin (lipopolysaccharides from *E. coli* 055:B5, LPS, Sigma-Aldrich, UK; i.v. dose of 100 ng/kg body weight). The dose of LPS had been determined previously to evoke a robust increase in plasma cortisol followed by a delay of in the LH surge onset (Fergani, et al. 2012). The timing of the treatments was chosen in order to precede all sexual behaviours and not just mounting. Two groups were killed at 31 h (31h control, n=6 and 31h LPS group, n=5) and two groups at 40 h after progesterone withdrawal (40h control, n=5 and 40h LPS group, n=5). Ewes were euthanised with pentobarbitone and perfused with: 2 litres 0.1M phosphate buffer (PB; pH 7.4) containing 25,000IU per litre of heparin and 1% sodium nitrate; then 2 litres Zamboni fixative (4 % paraformaldehyde) and 7.5 % saturated picric acid in 0.1M PB, pH 7.4); followed by 500 ml of the same fixative containing 30 % sucrose. Hypothalamic blocks (17 mm in width) were obtained (extending from the optic chiasma to the mammillary

bodies). Free-floating sections were stored in cryoprotectant solution and stored at -20 ^{0}C until processed for immunohistochemistry.

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

Dual-label immunofluorescence was carried out on 40 μm 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^{0}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 ^{0}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

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

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

162 Interestingly, we observed a different dynorphin cell activation pattern compared to what
163 we had previously reported for kisspeptin (Fergani, et al. 2013). In order to confirm this
164 discrepancy we performed triple-label immunohistochemistry for c-fos, kisspeptin and
165 dynorphin. This consisted of an immunoperoxidase protocol in which nuclear c-Fos was
166 detected first with diaminobenzidine as chromogen (DAB; brown reaction product) followed
167 by visualisation of kisspeptin and dynorphin with immunofluorescence. As kisspeptin and
168 dynorphin antibodies were both derived in the rabbit, we used a previously described modified
169 protocol (Cheng, et al. 2010, Hunyady, et al. 1996). Free-floating sections were washed
170 thoroughly in PBS for 2 h to remove the cryoprotectant solution followed by a 15 min
171 incubation in 40 % methanol and 1 % hydrogen peroxide (H_2O_2; 316989, Sigma-Aldrich, UK)
172 in PBS to inactivate endogenous peroxidases. Sections were then incubated for 1 h in blocking
173 solution (10 % donkey serum in PBS), followed by a 72 h incubation in rabbit anti-c-Fos
174 antibody (*1:5,000*) at 4 ^{0}C. After, sections were labelled with biotinylated donkey anti-rabbit
175 IgG (711-065-152, Jackson Immunoresearch West Grove, PA; *1:500*) for 2 h, followed by 90
176 min in Vectastain Elite ABC kit (*1:250* in PBS; PK6100, Vector Laboratories Ltd, UK). Nuclear
177 c-Fos was visualised by 5 min incubation in DAB (SK-4100, Vector Laboratories, Ltd, UK).
178 The second immunohistochemical procedure consisted of incubation for 72 h in rabbit anti-
179 kisspeptin (lot #564; gift from Prof. Alain Caraty, INRA Nouzilly, France; *1:150,000*) at 4 ^{0}C.
180 Following incubation, sections were labelled with biotinylated donkey anti-rabbit IgG (*1:500*;
181 for 2 h) and then incubated in Vectastain Elite ABC kit (*1:250*; for 90 min). The signal was
182 amplified in TSA for 10 min (*1:200*; New England Nuclear Life Science Products Life

Sciences, Boston, MA) diluted in PBS with 0.003 % H_2O_2 as substrate (Cheng, et al. 2010) and then labelled with streptavidin conjugated AlexaFluor 488 (S11223, Molecular Probes, Eugene, OR, USA; *1:100*) for 2 h. A third immunohistochemical procedure was then performed with a 72 h incubation with rabbit anti-Dynorphin (T-4268, Peninsula Laboratories, LLC, San Carlos, CA; *1:10,000*) and subsequent labelling with donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch West Grove, PA; *1:500*) for 2 h. Finally, sections were washed, mounted on chrome alum gelatin-coated slides, dried, and cover-slipped with Vectashield anti-fading mounting medium.

Data collection and analysis

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

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

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

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

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

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

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

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

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

Kisspeptin, dynorphin and NKB cell numbers and percentage co-localisation: In order to address the differential activation of kisspeptin and dynorphin neurones we analyzed the colocalisation between these two peptides throughout the follicular phase. Kisspeptin and dynorphin cell numbers were greater 'During sexual behaviour' ($P < 0.05$; Fig. 4A, 4B) 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 where 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 ERα (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

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

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

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

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

In the present study, acute LPS administration in the late follicular phase was accompanied by suppression of dynorphin and kisspeptin activation within the KNDy cell, a decrease in plasma oestradiol concentrations, and subsequent absence of a GnRH/LH surge. Several studies report down-regulation of the ARC kisspeptin system in rats and male rhesus monkeys 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.

Declaration of interest.

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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REFERENCES.

Advis, JP, J Klein, RO Kuljis, DK Sarkar, JM McDonald, and CA Conover 2003 Regulation of gonadotropin releasing hormone release by neuropeptide Y at the median eminence during the preovulatory period in ewes. *Neuroendocrinology* **77** 246-257.

Antonopoulos, J, GC Papadopoulos, AN Karamanlidis, and H Michaloudi 1989 Distribution of neuropeptides in the infundibular nucleus of the sheep. *Neuropeptides* **14** 121-128.

Billings, HJ, JM Connors, SN Altman, SM Hileman, I Holaskova, MN Lehman, CJ McManus, CC Nestor, BH Jacobs, and RL Goodman 2010 Neurokinin B acts via the neurokinin-3 receptor in the retrochiasmatic area to stimulate luteinizing hormone secretion in sheep. *Endocrinology* **151** 3836-3846.

Blache, D, CJ Fabre-Nys, and G Venier 1991 Ventromedial hypothalamus as a target for oestradiol action on proceptivity, receptivity and luteinizing hormone surge of the ewe. *Brain Res* **546** 241-249.

Caraty, A, C Fabre-Nys, B Delaleu, A Locatelli, G Bruneau, FJ Karsch, and A Herbison 1998 Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. *Endocrinology* **139** 1752-1760.

Caraty, A, DW, Miller, B, Delaleu, and GB, Martin 1997 Stimulation of LH secretion in sheep by central administration of corticotrophin-releasing hormone. *J Reprod Fertil* **111** 249-257.

Castellano, JM, VM Navarro, R Fernandez-Fernandez, R Nogueiras, S Tovar, J Roa, MJ Vazquez, E Vigo, FF Casanueva, E Aguilar, L Pinilla, C Dieguez, and M Tena-Sempere 2005 Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology* **146** 3917-3925.

Cheng, G, LM Coolen, V Padmanabhan, RL Goodman, and MN Lehman 2010 The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology* **151** 301-311.

Clarke, IJ, JT Cummins, ME Crowder, and TM Nett 1988 Pituitary receptors for gonadotropin-releasing hormone in relation to changes in pituitary and plasma gonadotropins in ovariectomized hypothalamo/pituitary-disconnected ewes. II. A marked rise in receptor number during the acute feedback effects of estradiol. *Biol Reprod* **39** 349-354.

Clarke, IJ, RJ, Horton, and BW, Doughton 1990 Investigation of the mechanism by which insulin-induced hypoglycemia decreases luteinizing hormone secretion in ovariectomized ewes. *Endocrinology* **127** 1470-1476.

Dobson, H, and RF Smith 2000 What is stress, and how does it affect reproduction? *Anim Reprod Sci* **60-61** 743-752.

Domanski, E, LK Chomicka, A Ostrowska, A Gajewska, and K Mateusiak 1991 Release of luteinizing hormone-releasing hormone, beta-endorphin and noradrenaline by the nucleus infundibularis/median eminence during periovulatory period in the sheep. *Neuroendocrinology* **54** 151-158.

Dufourny, L, A Caraty, IJ Clarke, JE Robinson, and DC Skinner 2005 Progesterone-receptive beta-endorphin and dynorphin B neurons in the arcuate nucleus project to regions of high gonadotropin-releasing hormone neuron density in the ovine preoptic area. *Neuroendocrinology* **81** 139-149.

Estrada, KM, S Pompolo, MJ Morris, AJ Tilbrook, and IJ Clarke 2003 Neuropeptide Y (NPY) delays the oestrogen-induced luteinizing hormone (LH) surge in the ovariectomized ewe: further evidence that NPY has a predominant negative effect on LH secretion in the ewe. *J Neuroendocrinol* **15** 1011-1020.

Fabre-Nys, C, D Blache, MR Hinton, JA Goode, and KM Kendrick 1994 Microdialysis measurement of neurochemical changes in the mediobasal hypothalamus of ovariectomized ewes during oestrus. *Brain Res* **649** 282-296.

Fabre-Nys, C, D Chesneau, C de la Riva, MR Hinton, A Locatelli, S Ohkura, and KM Kendrick 2003 Biphasic role of dopamine on female sexual behaviour via D2 receptors in the mediobasal hypothalamus. *Neuropharmacology* **44** 354-366.

Fabre-Nys, C, and H Gelez 2007 Sexual behavior in ewes and other domestic ruminants. *Horm Behav* **52** 18-25.

Fergani, C, JE Routly, DN Jones, LC Pickavance, RF Smith, and H Dobson 2013 Kisspeptin, c-Fos and CRFR type 2 expression in the preoptic area and mediobasal hypothalamus during the follicular phase of intact ewes, and alteration after LPS. *Physiol Behav* **110-111** 158-168.

Fergani, C, JE Routly, DN Jones, LC Pickavance, RF Smith, and H Dobson 2014 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. *Biol Reprod* **91** 141.

Fergani, C, AK Saifullizam, JE Routly, RF Smith, and H Dobson 2012 Estrous behavior, luteinizing hormone and estradiol profiles of intact ewes treated with insulin or endotoxin. *Physiol Behav* **105** 757-765.

Foradori, CD, M Amstalden, RL Goodman, and MN Lehman 2006 Colocalisation of dynorphin a and neurokinin B immunoreactivity in the arcuate nucleus and median eminence of the sheep. *J Neuroendocrinol* **18** 534-541.

Foradori, CD, RL Goodman, and MN Lehman 2005 Distribution of preprodynorphin mRNA and dynorphin-a immunoreactivity in the sheep preoptic area and hypothalamus. *Neuroscience* **130** 409-418.

Franceschini, I, D Lomet, M Cateau, G Delsol, Y Tillet, and A Caraty 2006 Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* **401** 225-230.

Ghuman, SP, R Morris, J Scherzer, JE Routly, A Mobasheri, RF Smith, and H Dobson 2011 Neuronal responses in the brainstem and hypothalamic nuclei following insulin treatment during the late follicular phase in the ewe. *Reprod Domest Anim* **46** 121-129.

Gonzalez, MI, V Kalia, DR Hole, and CA Wilson 1997 alpha-Melanocyte-stimulating hormone (alpha-MSH) and melanin-concentrating hormone (MCH) modify monoaminergic levels in the preoptic area of the rat. *Peptides* **18** 387-392.

Goodman, RL, I Holaskova, CC Nestor, JM Connors, HJ Billings, M Valent, MN Lehman, and SM Hileman 2011 Evidence that the arcuate nucleus is an important site of progesterone negative feedback in the ewe. *Endocrinology* **152** 3451-3460.

Goodman, RL, MN Lehman, JT Smith, LM Coolen, CV de Oliveira, MR Jafarzadehshirazi, A Pereira, J Iqbal, A Caraty, P Ciofi, and IJ Clarke 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* **148** 5752-5760.

Grachev, P, XF Li, and K O'Byrne 2013 Stress regulation of kisspeptin in the modulation of reproductive function. *Adv Exp Med Biol* **784** 431-454.

Grachev, P, KL Porter, LM Coolen, RB McCosh, JM Connors, SM Hileman, MN Lehman, and RL Goodman 2016 Surge-like LH secretion induced by retrochiasmatic area NK3R activation is mediated primarily by ARC kisspeptin neurones in the ewe. *J Neuroendocrinol*.

Helena, CV, N Toporikova, B Kalil, AM Stathopoulos, VV Pogrebna, RO Carolino, JA Anselmo-Franci, and R Bertram 2015 KNDy Neurons Modulate the Magnitude of the Steroid-Induced Luteinizing Hormone Surges in Ovariectomized Rats. *Endocrinology* **156** 4200-4213.

Herman, AP, A Krawczynska, J Bochenek, E Dobek, A Herman, and D Tomaszewska-Zaremba 2013 LPS-induced inflammation potentiates the IL-1beta-mediated reduction of LH secretion from the anterior pituitary explants. *Clin Dev Immunol* **2013** 926937.

Herman, AP, T Misztal, K Romanowicz, and D Tomaszewska-Zaremba 2012 Central injection of exogenous IL-1beta in the control activities of hypothalamic-pituitary-gonadal axis in anestrous ewes. *Reprod Domest Anim* **47** 44-52.

Hunyady, B, K Krempels, G Harta, and E Mezey 1996 Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *J Histochem Cytochem* **44** 1353-1362.

Iwasa, T, T Matsuzaki, M Murakami, F Shimizu, A Kuwahara, T Yasui, and M Irahara 2008 Decreased expression of kisspeptin mediates acute immune/inflammatory stress-induced suppression of gonadotropin secretion in female rat. *J Endocrinol Invest* **31** 656-659.

Kalra, SP 1993 Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. *Endocr Rev* **14** 507-538.

Kalra, SP, and WR Crowley 1992 Neuropeptide Y: a novel neuroendocrine peptide in the control of pituitary hormone secretion, and its relation to luteinizing hormone. *Front Neuroendocrinol* **13** 1-46.

Kalra, SP, MG Dube, A Sahu, CP Phelps, and PS Kalra 1991 Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. *Proc Natl Acad Sci U S A* **88** 10931-10935.

Karsch, FJ, JT Cummins, GB Thomas, and IJ Clarke 1987 Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing hormone in the ewe. *Biol Reprod* **36** 1207-1218.

Kasa-Vubu, JZ, GE Dahl, NP Evans, LA Thrun, SM Moenter, V Padmanabhan, and FJ Karsch 1992 Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone. *Endocrinology* **131** 208-212.

Kinsey-Jones, JS, XF Li, AM Knox, ES Wilkinson, XL Zhu, AA Chaudhary, SR Milligan, SL Lightman, and KT O'Byrne 2009 Down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r, mRNA expression is associated with stress-induced suppression of luteinising hormone secretion in the female rat. *J Neuroendocrinol* **21** 20-29.

Lehman, MN, LM Coolen, and RL Goodman 2010 Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* **151** 3479-3489.

Lehman, MN, FJ Ebling, SM Moenter, and FJ Karsch 1993 Distribution of estrogen receptor-immunoreactive cells in the sheep brain. *Endocrinology* **133** 876-886.

Lehman, MN, and FJ Karsch 1993 Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and beta-endorphin-immunoreactive neurons contain estrogen

receptors? A double-label immunocytochemical study in the Suffolk ewe. *Endocrinology* **133** 887-895.

Leranth, C, NJ MacLusky, M Shanabrough, and F Naftolin 1988 Catecholaminergic innervation of luteinizing hormone-releasing hormone and glutamic acid decarboxylase immunopositive neurons in the rat medial preoptic area. An electron-microscopic double immunostaining and degeneration study. *Neuroendocrinology* **48** 591-602.

Li, XF, AM Knox, and KT O'Byrne 2010 Corticotrophin-releasing factor and stress-induced inhibition of the gonadotrophin-releasing hormone pulse generator in the female. *Brain Res* **1364** 153-163.

Malven, PV, SA Haglof, and H Degroot 1992 Effects of intracerebral administration of neuropeptide-Y on secretion of luteinizing hormone in ovariectomized sheep. *Brain Res Bull* **28** 871-875.

Martin, P, Bateson, P. 1986 Measuring behaviour. Cambridge UK: Cambridge University Press.

Melis, MR, and A Argiolas 1995 Dopamine and sexual behavior. *Neurosci Biobehav Rev* **19** 19-38.

Merkley, CM, KL Porter, LM Coolen, SM Hileman, HJ Billings, S Drews, RL Goodman, and MN Lehman 2012 KNDy (kisspeptin/neurokinin B/dynorphin) neurons are activated during both pulsatile and surge secretion of LH in the ewe. *Endocrinology* **153** 5406-5414.

Murakawa, H, K. Iwata, T, Takeshita, and H, Ozawa 2016 Immunoelectron microscopic observation of the subcellular localization of kisspeptin, neurokinin B and dynorphin A in KNDy neurons in the arcuate nucleus of the female rat. *Neurosci Let* **26** 612-161-6.

Navarro, VM, JM Castellano, SM McConkey, R Pineda, F Ruiz-Pino, L Pinilla, DK Clifton, M Tena-Sempere, and RA Steiner 2011 Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab* **300** E202-210.

Navarro, VM, ML Gottsch, C Chavkin, H Okamura, DK Clifton, and RA Steiner 2009 Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* **29** 11859-11866.

674 **Naylor, AM, DW, Porter, and DW, Lincoln** 1990 Central administration of corticotrophin-
675 releasing factor in the sheep: effects on secretion of gonadotrophins, prolactin and
676 cortisol. *J Endocrinol* **124** 117-125.
677 **Pierce, BN, PH Hemsworth, ET Rivalland, ER Wagenmaker, AD Morrissey, MM**
678 **Papargiris, IJ Clarke, FJ Karsch, AI Turner, and AJ Tilbrook** 2008 Psychosocial
679 stress suppresses attractivity, proceptivity and pulsatile LH secretion in the ewe. *Horm*
680 *Behav* **54** 424-434.
681 **Pillon, D, A Caraty, C Fabre-Nys, and G Bruneau** 2003 Early decrease of
682 proopiomelanocortin but not neuropeptide Y mRNA expression in the mediobasal
683 hypothalamus of the ewe, during the estradiol-induced preovulatory LH surge. *Gen*
684 *Comp Endocrinol* **134** 264-272.
685 **Porter, DW, AM Naylor, AS McNeilly, and DW Lincoln** 1993 Endocrine actions of central
686 neuropeptide Y in the ewe: activation of the hypothalamo-pituitary-adrenal axis by
687 exogenous neuropeptide Y and role of endogenous neuropeptide Y in the secretion of
688 luteinizing hormone during the oestrous cycle. *J Neuroendocrinol* **5** 163-174.
689 **Porter, KL, SM Hileman, SL Hardy, CC Nestor, MN Lehman, and RL Goodman** 2014
690 Neurokinin-3 receptor activation in the retrochiasmatic area is essential for the full
691 pre-ovulatory luteinising hormone surge in ewes. *J Neuroendocrinol* **26** 776-784.
692 **Qi, Y, J Iqbal, BJ Oldfield, and IJ Clarke** 2008 Neural connectivity in the mediobasal
693 hypothalamus of the sheep brain. *Neuroendocrinology* **87** 91-112.
694 **Richter, TA, JE Robinson, JM Lozano, and NP Evans** 2005 Progesterone can block the
695 preovulatory gonadotropin-releasing hormone/luteinising hormone surge in the ewe
696 by a direct inhibitory action on oestradiol-responsive cells within the hypothalamus. *J*
697 *Neuroendocrinol* **17** 161-169.
698 **Robinson, JE, J Grindrod, S Jeurissen, JA Taylor, and WP Unsworth** 2010 Prenatal
699 exposure of the ovine fetus to androgens reduces the proportion of neurons in the
700 ventromedial and arcuate nucleus that are activated by short-term exposure to
701 estrogen. *Biol Reprod* **82** 163-170.
702 **Sahu, A, MG Dube, CP Phelps, CA Sninsky, PS Kalra, and SP Kalra** 1995 Insulin and
703 insulin-like growth factor II suppress neuropeptide Y release from the nerve terminals
704 in the paraventricular nucleus: a putative hypothalamic site for energy homeostasis.
705 *Endocrinology* **136** 5718-5724.

Scimonelli, T, F Medina, C Wilson, and ME Celis 2000 Interaction of alpha-melanotropin (alpha-MSH) and noradrenaline in the median eminence in the control of female sexual behavior. *Peptides* **21** 219-223.

Skinner, DC, and AE Herbison 1997 Effects of photoperiod on estrogen receptor, tyrosine hydroxylase, neuropeptide Y, and beta-endorphin immunoreactivity in the ewe hypothalamus. *Endocrinology* **138** 2585-2595.

Smith, JT 2009 Sex steroid control of hypothalamic Kiss1 expression in sheep and rodents: comparative aspects. *Peptides* **30** 94-102.

Smith, MJ, and RV Gallo 1997 The effect of blockade of kappa-opioid receptors in the medial preoptic area on the luteinizing hormone surge in the proestrous rat. *Brain Res* **768** 111-119.

Takumi, K, N Iijima, S Higo, and H Ozawa 2012 Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. *Neurosci Lett* **531** 40-45.

Taylor, JA, ML Goubillon, KD Broad, and JE Robinson 2007 Steroid control of gonadotropin-releasing hormone secretion: associated changes in pro-opiomelanocortin and preproenkephalin messenger RNA expression in the ovine hypothalamus. *Biol Reprod* **76** 524-531.

Thind, KK, and PC Goldsmith 1988 Infundibular gonadotropin-releasing hormone neurons are inhibited by direct opioid and autoregulatory synapses in juvenile monkeys. *Neuroendocrinology* **47** 203-216.

Wagenmaker, ER, KM Breen, AE Oakley, BN Pierce, AJ Tilbrook, AI Turner, and FJ Karsch 2009 Cortisol interferes with the estradiol-induced surge of luteinizing hormone in the ewe. *Biol Reprod* **80** 458-463.

Wahab, F, R Bano, S Jabeen, S Irfan, and M Shahab 2010 Effect of peripheral kisspeptin administration on adiponectin, leptin, and resistin secretion under fed and fasting conditions in the adult male rhesus monkey (Macaca mulatta). *Horm Metab Res* **42** 570-574.

Walsh, JP, and IJ Clarke 1996 Effects of central administration of highly selective opioid mu-, delta- and kappa-receptor agonists on plasma luteinizing hormone (LH), prolactin, and the estrogen-induced LH surge in ovariectomized ewes. *Endocrinology* **137** 3640-3648.

Whirledge, S, and JA Cidlowski 2013 A role for glucocorticoids in stress-impaired reproduction: beyond the hypothalamus and pituitary. *Endocrinology* **154** 4450-4468.

Zhang, Q, and RV Gallo 2003 Presence of kappa-opioid tone at the onset of the ovulatory luteinizing hormone surge in the proestrous rat. *Brain Res* **980** 135-139.

Figure Legends

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

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

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

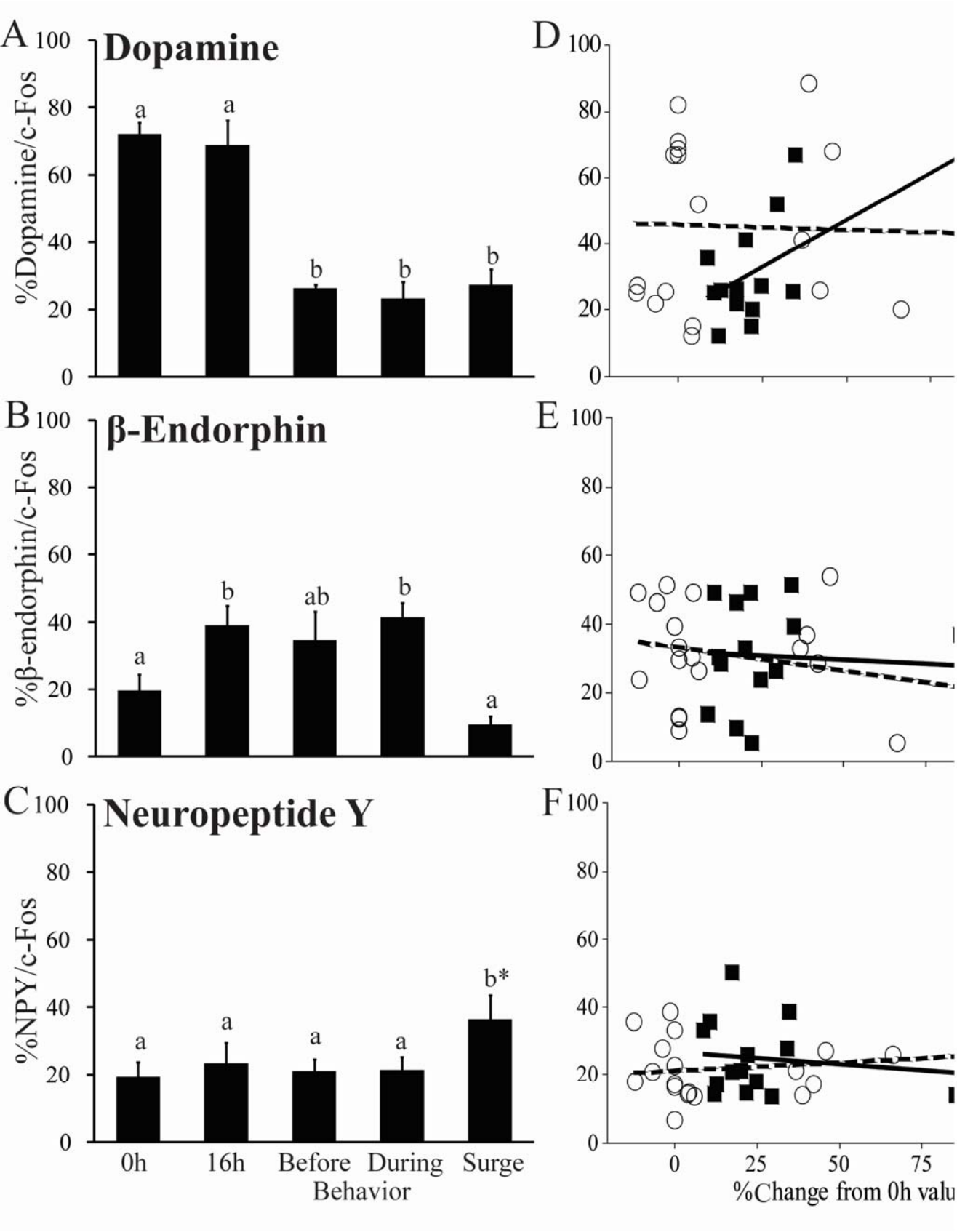
and NKB/c-Fos, respectively). Animals are grouped according to time as well as hormonal and behavioural status (for details, see Fig. 1 legend). Within each panel, differences between the percentages are indicated by different letters on top of each bar ($P < 0.05$). **D, E** and **F**: Regression graphs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

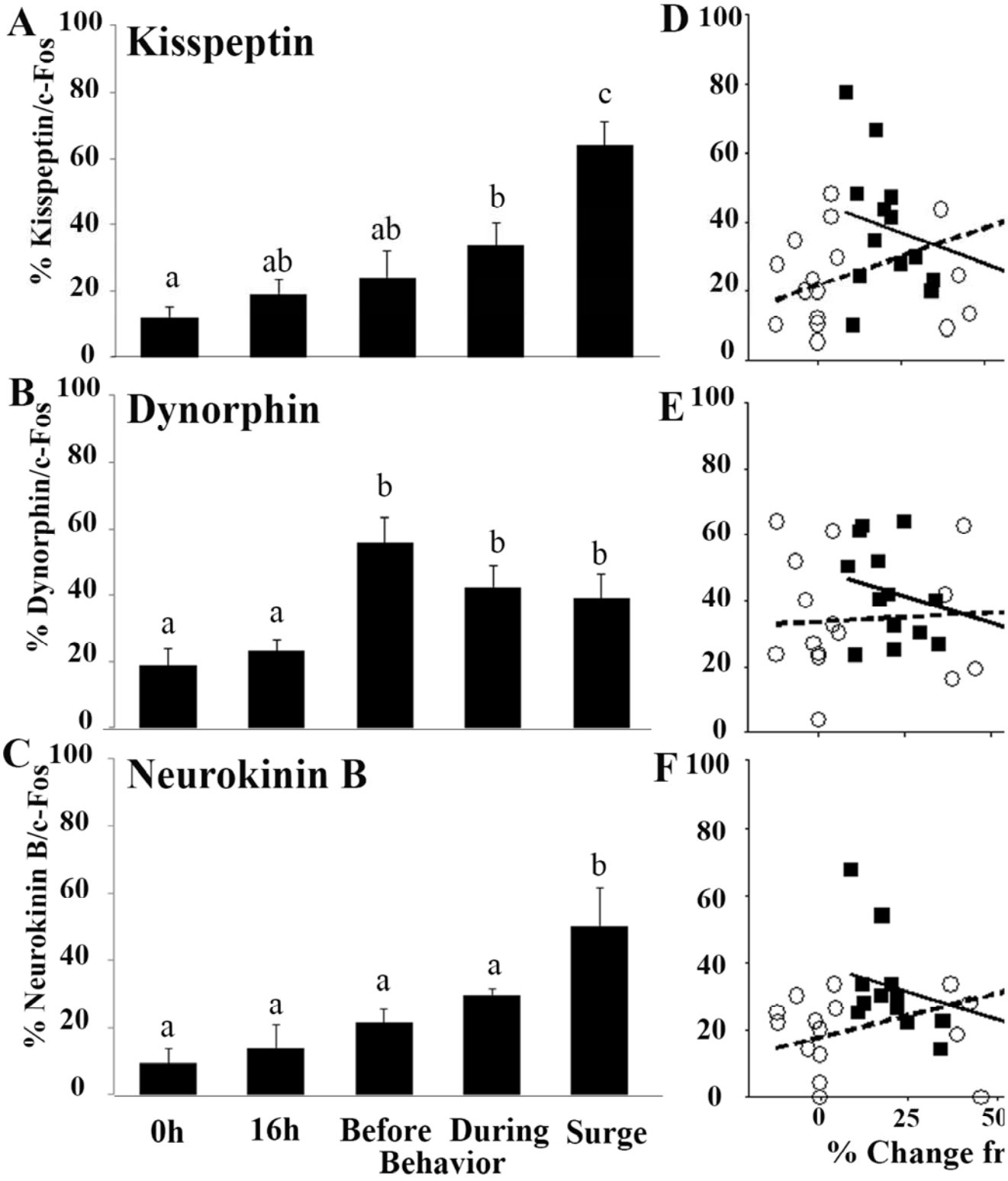
FIG. 4. A, B and **C**: Mean (±SEM) number of kisspeptin, dynorphin and NKB cells, respectively. **D**: mean % (±SEM) kisspeptin cells co-localising dynorphin, and **E**: mean % (±SEM) dynorphin cells co-localising kisspeptin. **F**: mean (±SEM) number of dynorphin (black bars) and kisspeptin (white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

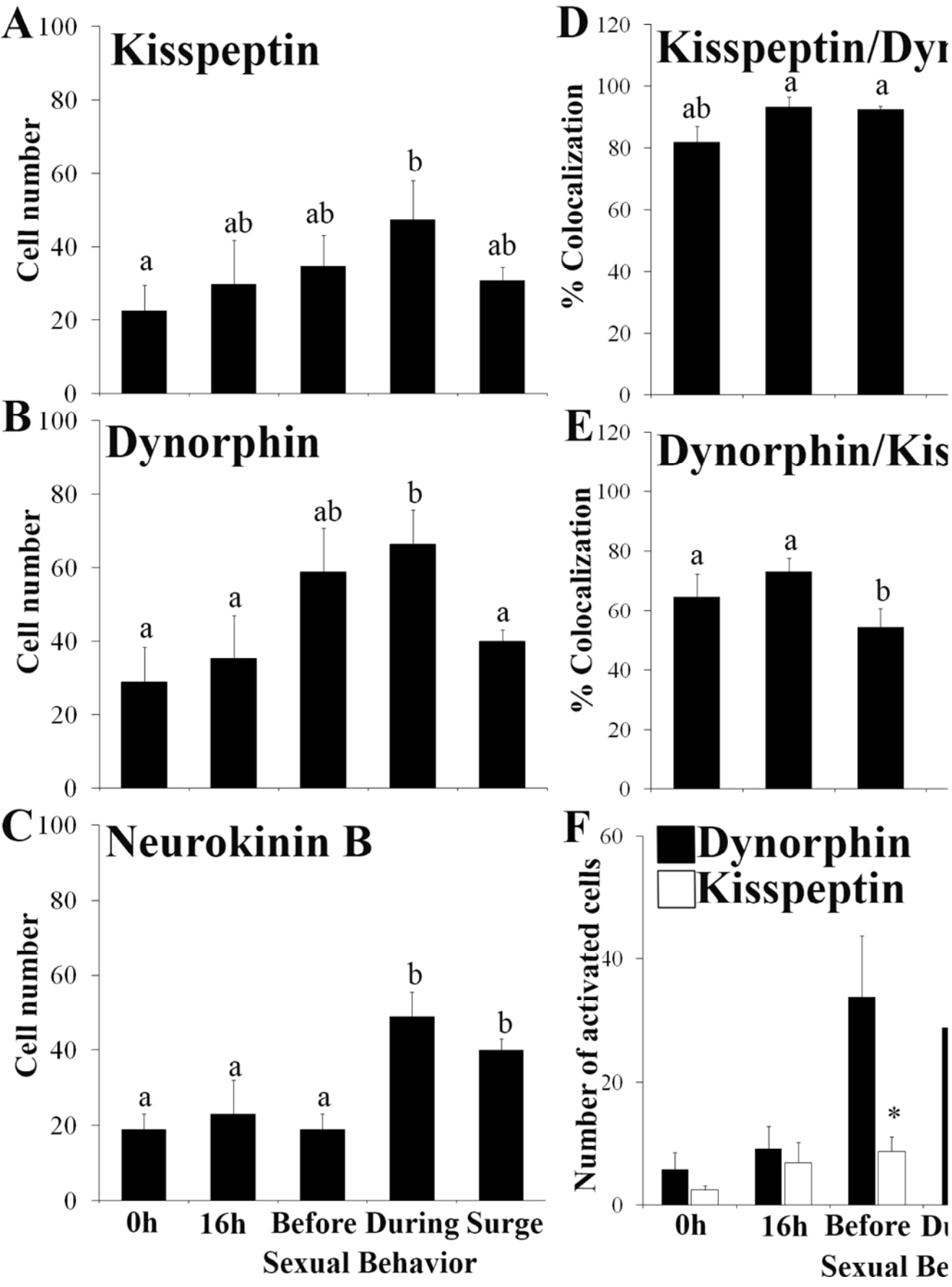
FIG. 5. A, B and **C**: Sets of photomicrographs in the ARC that were triple-labelled for c-Fos, kisspeptin and dynorphin, as well as a merged image (**D**) in control animals at 31 h after progesterone withdrawal (that is, before the onset of sexual behavior or the LH surge). Photomicrographs from the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

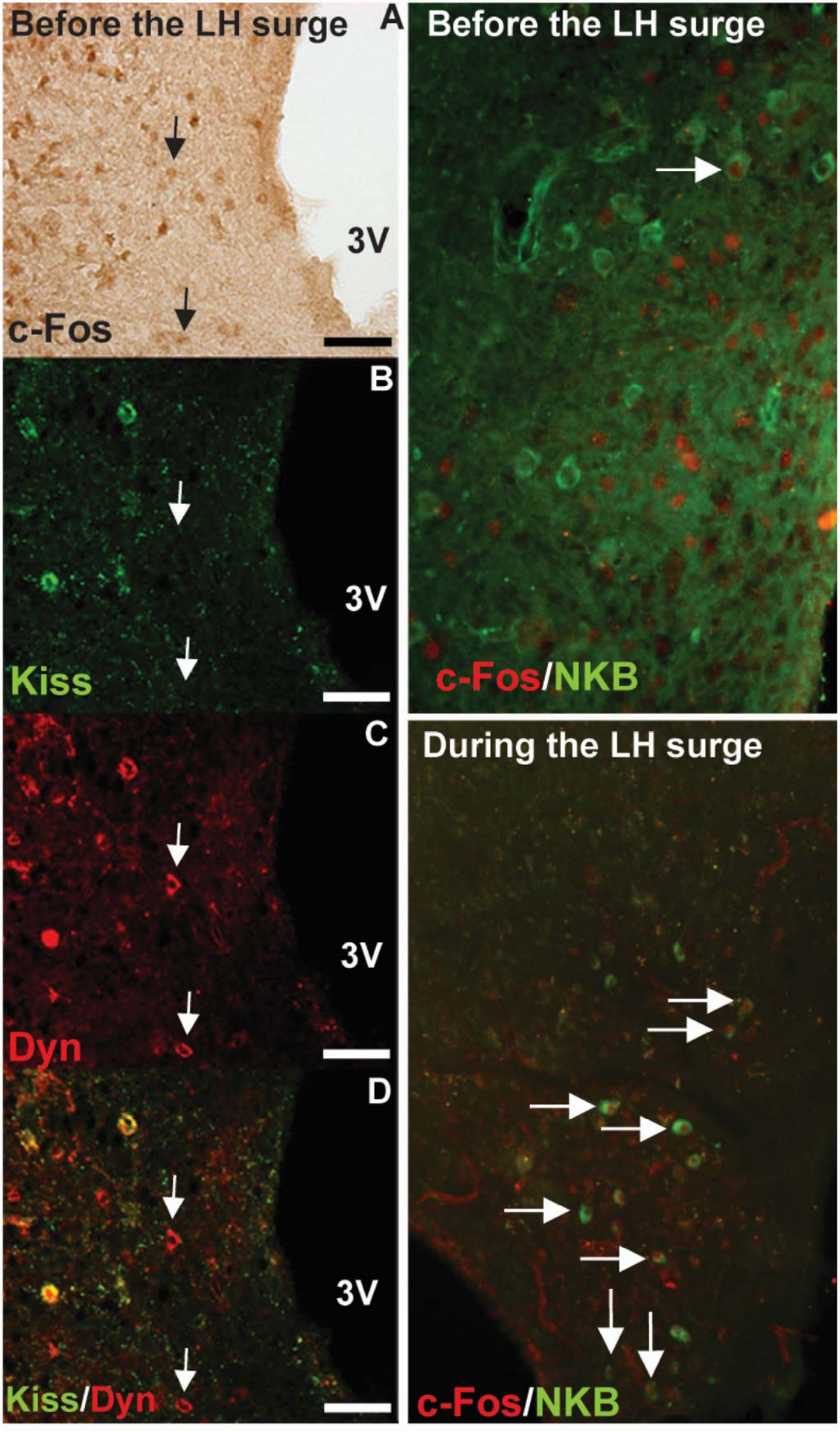
examples of single-labelled dynorphin cells co-localising c-Fos**. E-F**: Arrows indicate examples of dual-labelled cells. Original magnification: ×20 (A-E), original magnification: x10 (F). 3V = third ventricle.

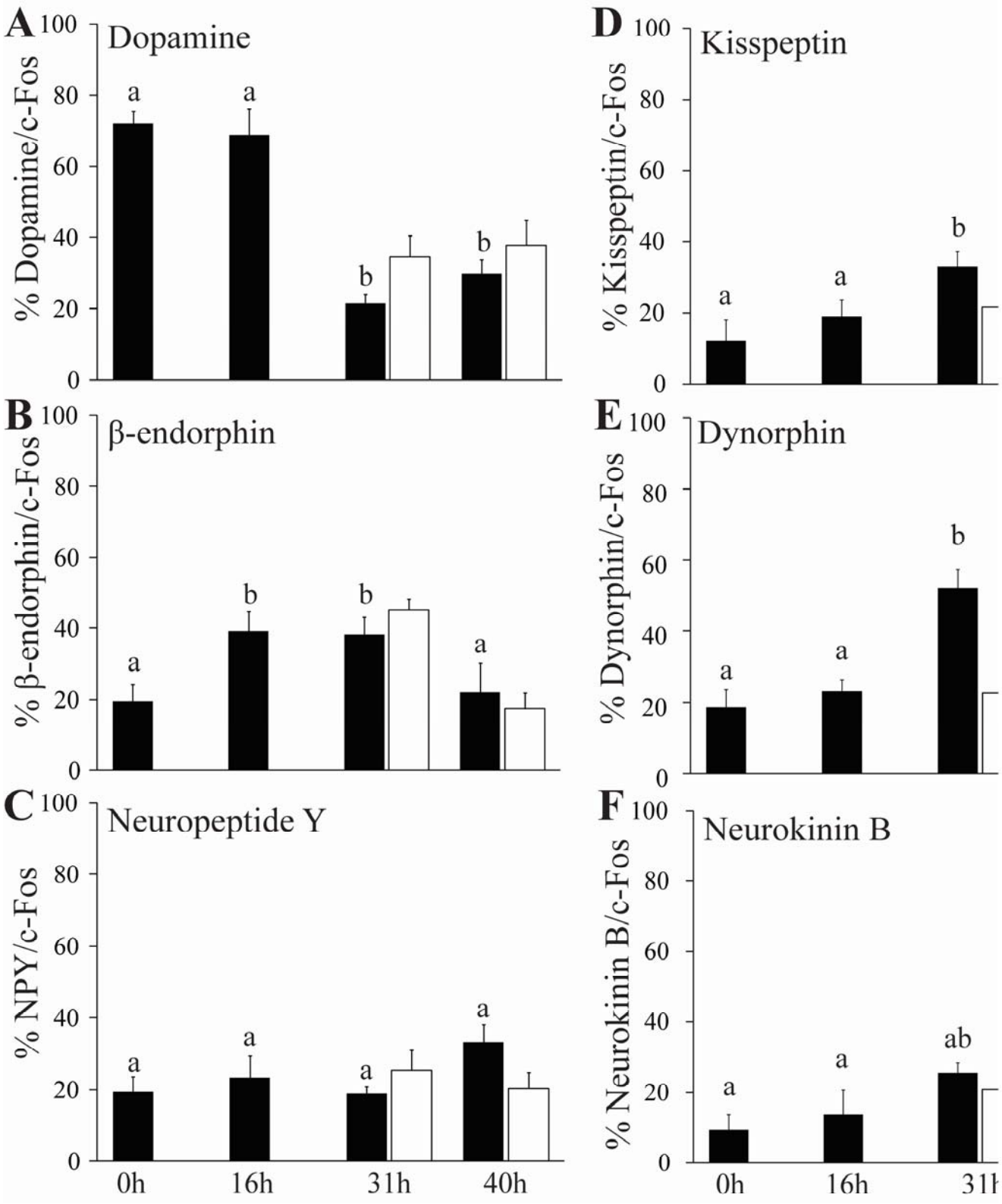
FIG. 6. A, B, C, D, E, F: Mean % (±SEM) dopamine, β-endorphin, neuropeptide Y, kisspeptin, dynorphin and NKB cells, respectively, that co-express c-Fos in the ARC across the follicular phase of control ewes as determined by immunofluorescence. Animals are grouped according to killing time after progesterone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


A
Dopamine
%Dopamine/c-Fos
100
80
60
40
20
0
a
a
b
b
b
B
β-Endorphin
%β-endorphin/c-Fos
a
b
ab
b
a
C
Neuropeptide Y
%NPY/c-Fos
a
a
a
a
b*
0h
16h
Before
During
Surge
Behavior
D
E
F
0
25
50
75
%Change from 0h valu


A
Kisspeptin
% Kisspeptin/c-Fos
100
80
60
40
20
0
a
ab
ab
b
c
B
Dynorphin
% Dynorphin/c-Fos
a
a
b
b
b
C
Neurokinin B
% Neurokinin B/c-Fos
a
a
a
a
b
0h
16h
Before
During
Surge
Behavior
D
E
F
0
25
50
% Change fr


A
Kisspeptin
Cell number
100
80
60
40
20
0
a
ab
ab
b
ab
B
Dynorphin
Cell number
a
a
ab
b
a
C
Neurokinin B
Cell number
a
a
a
b
b
0h
16h
Before
During
Surge
Sexual Behavior
D
Kisspeptin/Dyn
% Colocalization
120
100
80
60
40
20
0
ab
a
a
E
Dynorphin/Kis
% Colocalization
a
a
b
F
Dynorphin
Kisspeptin
Number of activated cells
60
40
20
0
*
0h
16h
Before
Du
Sexual Be


Before the LH surge
A
3V
c-Fos
B
3V
Kiss
C
3V
Dyn
D
3V
Kiss/Dyn
Before the LH surge
c-Fos/NKB
During the LH surge
c-Fos/NKB


A
Dopamine
% Dopamine/c-Fos
B
β-endorphin
% β-endorphin/c-Fos
C
Neuropeptide Y
% NPY/c-Fos
D
Kisspeptin
% Kisspeptin/c-Fos
E
Dynorphin
% Dynorphin/c-Fos
F
Neurokinin B
% Neurokinin B/c-Fos
0
20
40
60
80
100
a
b
ab
0h
16h
31h
40h