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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

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Abstract

Increasing estradiol concentrations during the late follicular phase stimulate sexual behavior and the GnRH/LH surge, and it is known that kisspeptin signaling is essential for the latter. Administration of LPS can block these events, but the mechanism involved is unclear. We examined brain tissue from intact ewes to determine: i) which regions are activated with respect to sexual behavior, the LH surge and LPS administration, ii) the location and activation pattern of kisspeptin cells in control and LPS treated animals, and iii) whether CRFR type 2 is involved in such disruptive mechanisms. Follicular phases were synchronized with progesterone vaginal pessaries and control animals were killed at 0h, 16h, 31h or 40h (n=4-6/group) after progesterone withdrawal (time zero). At 28 h, other animals received endotoxin (LPS; 100 ng/kg) and were subsequently killed at 31h or 40h (n=5/group). LH surges only occurred in control ewes, during which there was a marked increase in c-Fos expression within the ventromedial nucleus (VMN), arcuate nucleus (ARC), medial preoptic area (mPOA), as well as an increase in the percentage of kisspeptin cells co-expressing c-Fos in the ARC and mPOA compared to animals sacrificed at all other times. Expression of c-Fos also increased in the bed nucleus of the stria terminalis (BNST) in animals just before the expected onset of sexual behavior. However, LPS treatment increased c-Fos expression within the VMN, ARC, mPOA and diagonal band of broca (dBb), along with CRFR type 2 immunoreactivity in the lower part of the ARC and median eminence (ME), compared to controls. Furthermore, the percentage of kisspeptin cells co-expressing c-Fos was lower in the ARC and mPOA. Thus, we hypothesize that in intact ewes, the BNST is involved in the initiation of sexual behavior while the VMN, ARC, mPOA as well as kisspeptin cells located in the latter two areas are involved in estradiol positive feedback only during the LH surge. By contrast, disruption of sexual behavior and the LH surge after LPS involves cells located in the VMN, ARC, mPOA and dBb, as well as cells containing CRFR type 2 in the lower part of the ARC and ME, and is accompanied by inhibition of kisspeptin cell activation in both the ARC and mPOA.

Introduction

In the late follicular phase, decreasing progesterone and increasing estradiol concentrations trigger the onsets of sexual behavior and the GnRH/LH (gonadotropin releasing hormone/luteinizing hormone) surges (1). However, sudden activation of the hypothalamic-pituitary-adrenal axis by an immune/inflammatory challenge, in the form of E. coli lipopolysaccharide (LPS), lowers plasma estradiol concentrations and delays the onsets of sexual behavior and the LH surge of intact ewes via unknown mechanisms (2).

In the normal follicular phase, it is generally accepted that the major feedback effects of estradiol on GnRH secretion occur indirectly. Estradiol acts upon estradiol-receptive neurons, located in the hypothalamus/preoptic area (POA; 3-4), with axons that project directly and/or indirectly to GnRH neurons (5). However, the precise location and cell phenotypes involved at different stages of the follicular phase remain unknown with respect to either change in sexual behavior or GnRH secretion. A possible scenario is that alterations in the progesterone:estradiol balance could influence cellular activation patterns in specific brain areas during progressive stages of the follicular phase. Monitoring the presence of c-Fos (a marker for neuronal activation; 6) would pinpoint the location of activated cells involved in sexual behavior and GnRH/LH surge generating mechanisms. By contrast, we hypothesize that the physiological activation patterns are altered by LPS, and c-Fos will also reveal the regions through which this stressor acts to disrupt the follicular phase.

A plethora of recent studies provide evidence that the hypothalamic neuropeptide kisspeptin is essential for GnRH/LH surge release across a large number of species (7-8). In the ewe, kisspeptin has been implicated in mediating estradiol negative (9) and positive feedback (10-12), as well as orchestrating GnRH/LH pulsatility (8) and surge secretion (10-15). However, the pattern of kisspeptin cell activation in both main populations of cells (ARC and mPOA) during the whole course of the follicular phase has not yet been investigated. Interestingly, in addition to steroid hormones, kisspeptin neurons have been proven sensitive to the actions of various stressors,

including LPS, and are likely to operate as transmitters for effects on GnRH neurons (16). Specifically, a few studies in female rats report reduced levels of kisspeptin mRNA and suppression of the reproductive axis after a challenge with LPS (17-18). To date, there are no studies addressing the potential alterations of kisspeptin cell activation (at peptide level) in key hypothalamic nuclei during acute inflammatory stress in any species. Given the crucial role of kisspeptin in regulating GnRH secretion, we hypothesize that stress-induced suppression of reproductive parameters is accompanied by a failure of kisspeptin cell activation.

Regarding the potential mechanisms involved in the disruption of sexual behavior and GnRH/LH patterns after LPS, various studies suggest that a common pathway for the action of stressors is to stimulate the cellular activity of the paraventricular nucleus (PVN), especially neurons secreting corticotropin releasing factor (CRF; 18-20). In the rat, CRF has a pivotal role in stress-induced suppression of GnRH pulses and is thus a prime candidate for transmitting the 'stress' signal to GnRH cells directly or via interneurons (21). Li *et al.,* (22) report that LPS involves the activation of Type 2 but not Type 1 CRF receptor (CRFR) to mediate inhibitory actions. However, the importance of CRFR has not been extensively investigated in the ewe.

In the present study we examined brain tissue from intact ewes sacrificed at various times during the follicular phase with or without the administration of LPS. We used c-Fos to locate the brain areas that exhibit activation events before or during the onset of sexual behavior, during the LH surge or after LPS and may, therefore, be involved in the physiological and/or pathological mechanisms. Furthermore, we aimed to map the pattern of kisspeptin cell activation (by measuring co-expression with c-Fos; 6) in the ARC and mPOA at various times during the follicular phase, as well as testing the hypothesis that the LH surge disruption after the application of LPS is associated with inhibition of kisspeptin cell activation. Finally, we examined the presence of CRFR Type 2 in the ARC and ME to establish whether up-regulation of this particular receptor may play a role in the LH surge inhibition after stress.

Materials and Methods

Animals, study design and blood sampling procedure

The study was performed in the mid-breeding season (October/November) on 30 mature intact Lleyn crossbred ewes. From two weeks prior to the study, the ewes were penned altogether indoors (space 15 x 7 meters) with the same 3 experienced teaser rams present throughout, to avoid spurious behavioural activity. Frequent handling for at least a week ensured that the animals were acclimatized to human contact. All procedures were conducted within requirements of the UK Animal (Scientific Procedures) Act, 1986, and approved by the University of Liverpool Animal Welfare Committee.

Ovarian follicular phases were synchronized with two intravaginal progesterone-releasing pessaries (Controlled Internal Drug Release [CIDR-G]; InterAg, Hamilton, New Zealand) for nine days and an intramuscular (i.m.) injection of prostaglandin (Lutalyse, 5 mg/ewe, Pharmacia & Upjohn, UK) 12 h before, and a second injection, at CIDR-G removal. The time of progesterone withdrawal (i.e., commencement of the follicular phase) is referred to hereafter as time 0 h.

The experimental protocol is outlined in Fig.1. The ewes were randomly allocated to six groups. One group was killed at 0 h (0 h control group; n=5) and another group at 16 h after progesterone withdrawal (16 h control group; n=4). At 28 h, the remaining animals received 2 ml saline vehicle or endotoxin (Lipopolysaccharides from Escherichia coli 055:B5, LPS, Sigma-Aldrich, UK; i.v. dose of 100 ng/kg body weight). The LPS dose is routinely used in our studies and evokes a robust cortisol increase and attenuation of the LH surge without other undue clinical effects (2). The timing of the treatments was chosen in order to precede all sexual behaviors and not just mounting. Two groups were killed at 31h (31h control, n=6 and 31h LPS group, n=5) and two groups at 40h after progesterone withdrawal (40h control, n=5 and 40h LPS group, n=5).

Frequent blood sampling, as well as the administration of all substances, was facilitated by insertion of a silastic catheter into the jugular vein of each ewe under local anesthesia before progesterone withdrawal. Patency was maintained with heparinized saline (Multihep, 100 iu/ml, Leo Laboratories, Princes Risborough, UK) after each blood withdrawal. Blood samples (5 ml at 0h, 16h, 24h and subsequently at 2h intervals till 40h) were collected and centrifuged immediately at 1000 *g* for 20 min at 4^0C. Plasma was stored at -20^0C until analysis. Samples, in duplicate, were analyzed by Enzyme-Linked Immunosorbent Assays (ELISAs) for LH, pregnane metabolites (equivalent to, and hereafter referred to as, progesterone) or cortisol. These assays were performed with methods adapted from (23-25), respectively.

LH results were expressed as ng equivalent of NIAMDD ovine LH 21 per ml plasma. Estradiol was measured with a modified radioimmunoassay (RIA) using 0.5 ml plasma extracted with 3 ml diethyl ether followed by evaporation to dryness. All assays were verified for use in sheep (26). Contemporary inter-assay and intra-assay coefficients of variation for LH, progesterone, cortisol and estradiol were all less than 12%. The minimum detectable amounts were 0.02 ng/ml; 0.16 ng/ml, 0.8 ng/ml and 0.2 pg/ml and assay precisions (in the mid-range of the standard curve) were 0.1 ng/ml, 0.01 ng/ml, 0.2 ng/ml and 0.2 pg/ml, respectively. All samples from individual animals were measured in the same assay for each hormone.

Visual observation of sexual behavior

Ewe and ram sexual behavior was recorded by two trained observers for a 30-minute observation period prior to each blood sample collection. The observers were placed in an elevated position to assist efficient monitoring of the entire pen where the animals were allowed to move freely. Once a minute throughout each observation period, it was noted if a ewe was within one meter of a ram [behavioral scan sampling; Martin and Bateson (27)]. In addition, the following behavioral signs were noted throughout each 30 min observation period: ram nosing the perineal region of the ewe; ewe being nudged by the ram without moving away; and, mounting of the ewe by the ram without moving away. Due to the 2-hourly observation regime, the

beginning/end of periods were, respectively, defined as the first/last (minus/plus 1.0h) 30-min observation period the animal exhibited a particular behavioral sign.

Tissue collection

Euthanasia was carried out with 20 ml 20% w/v sodium pentobarbitone (Pentobarbital, Loveridge, Southampton, UK), containing 25,000IU heparin. The solutions used for perfusion were: 2 liters 0.1M phosphate buffer (PB; pH7.4) containing 25,000IU per liter of heparin and 1% sodium nitrate; then 2 liters Zamboni fixative (4% paraformaldehyde) and 7.5% saturated picric acid in 0.1M PB, pH7.4); followed by 500ml of the same fixative containing 30% sucrose. The brain was left within the skull for a further 4h and then 500ml wash–out solution (0.1M PB, 40% sucrose and 0.1% sodium azide) was pumped through. Hypothalamic blocks were obtained and then frozen using isopentane (2-Methylbutane, Chromasolv®, for HPLC, Sigma-Aldrich, UK) and liquid nitrogen as described by Rosene *et al.,* (28) and stored at -80^{0}C. Frozen coronal sections (40 μm) were cut using a freezing microtome (Microm HM400R, Walldorf, Germany). Free-floating sections were stored in cryoprotectant solution (29) and stored at -20^{0}C until processed for immunohistochemistry.

c-Fos and kisspeptin dual-label immunohistochemistry

A series of every 15th section (600μm intervals) through the preoptic area and mediobasal hypothalamus was processed using a dual-immunoperoxidase protocol in which nuclear c-Fos was detected first with nickel sulfate-enhanced diaminobenzidine as chromogen (ni-DAB; black), followed by detection of cytoplasmic kisspeptin using unenhanced diaminobenzidine (DAB; brown). All steps were performed at room temperature unless otherwise stated. Antibodies were diluted with 2.5% normal donkey serum (catalogue item S2170, Biosera, UK), 1% Triton X-100 (T9284, Sigma-Aldrich, UK) and 0.25% sodium azide (Sigma- Aldrich, UK) in 0.1M phosphate buffer saline, pH7.2 (PBS). Free-floating sections were washed thoroughly in PBS for 2h to remove the cryoprotectant solution followed by a 15min incubation in 40% methanol and 1% hydrogen peroxide (H_2O_2; 316989, Sigma-Aldrich, UK) in PBS to inactivate endogenous peroxidases. After three 10min washes,

sections were incubated for 1h in blocking solution (10% donkey serum in PBS). This was followed by 72h incubation in rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA; 30) at 1:5000 at 4^0C. After incubation with primary antiserum, sections were washed thoroughly and incubated with biotinylated donkey anti-rabbit IgG (1:500; 711-065-152, Jackson Immunoresearch, West Grove, PA) for 2h, followed by three 10min washes and then 90min in Vectastain Elite ABC kit (1:250 in PBS; PK6100, Vector Laboratories Ltd, UK). After repeating washes, nuclear c-Fos was visualized by 5min incubation in ni-DAB (SK-4100, Vector Laboratories Ltd, UK). A second immunohistochemical procedure was then performed, as described above, using rabbit anti-kisspeptin serum (1:25,000; lot 564; gift from Prof. Alain Caraty, Nouzilly, France; 31), incubated for 72h at 4^0C and then visualized using DAB. Negative control sections were performed routinely by omitting primary antibody(s). This resulted in complete loss of staining.

CRFR Type 2 single-label immunohistochemistry

For CRFR Type 2 (1:4000; ab12964; Abcam UK; 32) three sections, 240μm apart, containing the ARC and ME were chosen and a staining protocol performed as described above. The exclusion of the primary or secondary antibody resulted in complete absence of staining.

Data analysis

Hormone, behavior and immunohistochemistry data were analyzed with Minitab® 15 statistical package (MINITAB Inc, Pennsylvania, USA). Results are expressed as mean ± SEM, and for all analyses, statistical significance was regarded when $P<0.05$. An LH surge was defined as a sustained increase (>4h) in LH plasma concentrations and onset was considered when the first value increased more than ten times the minimum detectable concentration (i.e., >10 ng/ml). Cortisol, progesterone and estradiol concentrations were compared between groups, per hour, with a general linear model (GLM) ANOVA followed by Tukey's multiple comparisons *post hoc* test, when appropriate. In addition, because of considerable variation, progesterone concentrations at 28h (i.e., just prior to treatment) were compared within groups to

the two mean consecutive maximum values recorded after treatment with a Wilcoxon sign rank test.

Sections were examined using a microscope (Nikon Microscope, Eclipse 80i) and photographed with a Nikon camera using a 20× objective. The areas examined were [as defined by Welento *et al.,* (33); Fig 6G-I]: the VMN (4 photographs per section, 6 sections per ewe), ARC (3 photographs per section, 10 sections per ewe, which consisted sections from the rostral, middle and caudal divisions of the nucleus), ME and bed nucleus of the stria terminalis (BNST; 1 photograph per section, 6 sections per ewe, for both areas), mPOA (2 photographs per section, 5 sections per ewe), diagonal band of Brocca (dBb; 2 photographs per section, 3 sections per ewe) and the PVN (1 photograph per section, 6 sections per ewe). CRFR Type 2 data were derived from 3 photographs that included the lower part of the ARC and the ME. Data from these two areas were combined due to the confined location of the receptors; i.e., on the 'border' either side of the ARC and ME boundary. All photographs were imported into Image J version 1.42q, and counts performed using the cell count plug-in. The observer was unaware of the animal identity and group. The mean total number and percentage of single- or dual-labeled cells was summed from the photographs of each area/section and then averaged for each ewe and compared with GLM ANOVA followed, where appropriate, by Tukey's multiple comparisons *post hoc* test. Means (±SEM), as presented in figures and results, were calculated by averaging mean values from ewes in each group.

Results

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

Plasma hormone profiles: Estradiol, Progesterone and Cortisol

In control animals, plasma estradiol concentrations continued to increase from 28h after PW to maximum values between 32h and 36h after PW (Fig 2A). However, after treatment with LPS, estradiol concentrations tended to be lower than controls 8h after treatment (i.e., at 36h after PW; $P<0.07$; Fig.2A) and, thereafter, continued to decrease significantly until ewes were killed at 40h ($P<0.02$; Fig 2A).

All groups had similar plasma progesterone concentrations at 0h and 28h after PW (33.7 ± 2.0 ng/ml and 6.6 ± 0.4 ng/ml, respectively). There was considerable between-animal variation, and values between treated and control groups were not different at each time point after treatment; therefore, a within-group comparison was also made. Within-group controls had similar concentrations of progesterone before and after treatment (Fig. 2B). Progesterone concentrations were different within the 40h LPS group, increasing from 6.9 ± 1.0 ng/ml to a mean maximum of 9.9 ± 1.6 ng/ml after treatment ($P<0.05$; Fig. 2B). Progesterone concentrations after PW within control animals are also shown in Fig. 2A.

In all control animals, mean plasma cortisol concentrations remained low throughout (10.5 ± 0.7 ng/ml; Fig. 2C). Before the application of LPS (i.e., at 24 and 28h after PW), cortisol concentrations did not differ from values in controls (Fig. 2C). At 30h, both groups of LPS animals had higher values compared to control groups ($P<0.001$; Fig. 2C). In the 40h LPS group, ewes had increased concentrations compared to the controls from 32h to 40h after PW ($P<0.05$ for all; Fig. 2C). Mean maximum cortisol concentrations (157 ± 19.8 ng/ml) for the LPS groups were observed 2h after treatment (Fig. 2C).

Luteinising hormone (LH) and behavioral profiles.

The onset of sexual behavior and the LH surge of individual ewes with respect to progesterone withdrawal (PW) are shown in Table 1. Control animals began exhibiting sexual behavior at 28.5 ± 2.4 h, and three of the five animals in the 40h-control group had an LH surge with a mean onset at 36.7 ± 1.3 h (Table 1). Three of the eight LPS treated animals exhibited sexual behavior, whereas none of the treated animals began an LH surge during 40h of study (Table 1). Subsequent data were

analyzed in two ways: the first consisted of only control ewe data, grouped according to time after PW, and incorporating sexual behavioral status and whether an LH surge had occurred; i.e., those killed: at 0h and 16h after PW; at 31h after PW but before the onset of sexual behavior (Before sexual behavior, n=3); at 31h or 40h after PW and during exhibition of sexual behavior but before an LH surge (During sexual behavior, n=5); or after the onset of sexual behavior and during the LH surge (Surge, n=3). This grouping was used to pinpoint the location of cells involved in sexual behavior and GnRH/LH surge generating mechanisms in control animals. Secondly, control and treated animal data were grouped according to time of killing after PW, and this was used to compare treatment effects.

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

c-Fos activation

The number of c-Fos positive cells in the VMN and mPOA was three times greater, and in the ARC four times greater, in the 'Surge' group compared to all other stages in the follicular phase of control ewes ($P<0.05$ for all comparisons; Fig 3A,B,C).

The number of c-Fos positive cells in the ME and dBb was not different at any stage (Fig 3D and 3E). However, the number of c-Fos positive cells in the BNST was three times greater in the 'Before sexual behavior' group ($P<0.001$ for all comparisons; Fig 3F).

Kisspeptin distribution and c-Fos co-expression

Kisspeptin immunoreactive cell bodies and fibers were found in anatomical structures as defined by Welento *et al.,* (33) and similar to those in previous reports (31): i.e., the rostral, but primarily medial and caudal ARC, extending to the pre-mammillary recess; the mPOA at the level of the organum vasculosum of the lamina terminalis; and the PVN (~ 30 cells per section and numerous fibers). A small number of scattered kisspeptin positive cells and fibers were detected alongside the third ventricle walls, the VMN and the dorsomedial hypothalamus but this was not consistent in all animals. A few kisspeptin cells (~ 5-10 cells per section) were observed in the ME internal zone, along with a dense fiber network, but this was less

dense in the external zone. No kisspeptin cells were observed in the dBb or the BNST.

The number of kisspeptin cells that co-expressed c-Fos in the ARC and mPOA increased in the 'Surge' group compared to all other stages in the follicular phase of control ewes ($P<0.05$ for both; Fig 4A and 4B).

Comparison of control and LPS treated ewes

c-Fos activation

At 31h and 40h after PW (i.e., 3h and 12h after LPS administration, respectively), there was a marked increase in the number of c-Fos positive cells in the VMN of LPS treated ewes, compared to controls ($P<0.05$; Fig 5A).

At 31h after PW, there was a marked increase in the number of c-Fos positive cells in the ARC of LPS treated ewes ($P<0.01$; Fig 5B) compared to controls. At 40h after PW, when the majority of control animals were undergoing an LH surge, control and LPS group data were not different (Fig 5B).

At 31h after PW, the number of c-Fos positive cells in the mPOA increased in the LPS group ($P<0.03$; Fig 5C) compared to controls. At 40h after PW, when the majority of control animals were undergoing an LH surge, control and LPS data were not different (Fig 5C).

The number of c-Fos positive cells in the ME and BNST was not altered by LPS (Fig 5D and 5F). However, at 31h and 40h after PW (i.e., 3h and 12h after treatment) LPS groups had markedly increased numbers of c-Fos positive cells in the dBb compared to controls ($P<0.05$; Fig 5E).

Kisspeptin and c-Fos co-expression

The total number of kisspeptin positive cell bodies varied among animals but was not altered with the application of LPS (Table 2).

In the ARC and mPOA at 31h after PW (i.e., 3h after LPS administration), the percentage of kisspeptin cells that co-expressed c-Fos in the LPS group was not

different to controls (Fig 6A and 6B). However, at 40h after PW (i.e., 12h after LPS), the percentage of kisspeptin cells that co-expressed c-Fos was markedly lower in LPS treated animals compared to controls in both areas ($P<0.01$; Fig 6A and 6B). Photomicrographs of sections of the ARC in control and LPS treated animals are shown in Fig 6C-F, with an indication of the areas taken and considered for analysis in Fig 6G-I.

c-Fos activation in the PVN and CRFR type 2 in the lower part of the ARC and ME.

The number of c-Fos positive cells in the PVN was not different between all control groups (Fig 7A). A marked increase in c-Fos positive cells was observed in the PVN after LPS treatment (31h and 40h LPS groups; $P<0.05$ for both times; Fig 7A and 7B) compared to control groups.

Immunohistochemistry revealed CRFR type 2 immunoreactivity in the lower part of the ARC, as well as the internal zone of the ME. At cellular level, the receptors had a 'ring-like' morphology and were cytoplasmic in nature (Fig 7D). There was no difference in the number of CRFR type 2 cells between control animals (Fig 7C). However, LPS increased CRFR type 2 immunoreactivity in the lower ARC + ME ($P<0.001$; Fig 7C). This was evident at 31h and 40h after PW.

Discussion

The LH surge (and not earlier stages of the follicular phase) of intact ewes was accompanied by an intense cellular activation within the VMN, ARC and mPOA, as well as an activation of kisspeptin cells located in the ARC and mPOA. This suggests that the above hypothalamic regions as well as both major populations of kisspeptin cells contribute to estradiol positive feedback to stimulate the GnRH/LH surge in the ewe. By contrast, LPS treatment in the late follicular phase was accompanied by: a) disruption of the LH surge, b) intense cellular activation within the VMN, ARC, mPOA, PVN and dBb at different times compared to controls, c) failure of kisspeptin cells to be activated in the ARC and mPOA, and d) an increase in CRFR Type 2 immunoreactivity in the lower part of the ARC and ME. The results of the present

study extend our knowledge of the GnRH/LH surge mechanism, and its disruption after stress, by demonstrating that the patterns of cellular activation in the hypothalamus during the course of the follicular phase or following an acute stressor differ in a region and time specific manner.

Cellular activation during the follicular phase of controls.

Estradiol implants in the mediobasal hypothalamus (MBH; the vicinity of the ARC and VMN) initiated sexual behavior and the GnRH surge (3-4), whereas the mPOA, dBb and ME are areas rich in ERα (34) and/or GnRH cell perikarya and fibers (35). Therefore, these hypothalamic regions are all prime candidates for the control of follicular phase events. We detected a marked activation within the VMN, ARC and mPOA during the LH surge compared to all other stages in the natural follicular phase. As the GnRH surge generating mechanism consists of three phases (activation, transmission and surge secretion; 36), our results suggest that cells located in the VMN, ARC and mPOA are activated only in the surge secretion phase and presumably facilitate GnRH neurosecretion at that time. This concurs with Richter *et al.,* (37) who examined ovariectomized (OVX) animals during the activation phase of the surge generating mechanism as well as during surge onset. They also found greater activation of the ARC and mPOA in the latter stage but not the former.

By contrast, the onset of sexual behavior did not cause any region-specific changes in hypothalamic c-Fos expression. However, an increase in BNST activation was observed in animals just before the expected onset of sexual behavior. Indeed, the BNST is an ERα-rich area (38) that receives projections from the cortical and medial nuclei of the amygdala which, in the ewe, are involved in the processing of olfactory information relevant to social recognition (39) and also sends projections to the mPOA where most GnRH cells are located (40-41). The BNST could, therefore, be an intermediary between the amygdala and the mPOA, transmitting pheromonal signals to GnRH neurons for the initiation of sexual behavior. It must be noted that the lack of activation in the key areas examined prior to the GnRH surge and sexual behavior does not exclude their involvement in the generation mechanism of these events. It may be that the phenotype of activated cells changes to stimulate sexual behavior

and the GnRH surge, even though the overall number of c-Fos activated cells remains the same. Interestingly, at the time when estradiol was reaching maximum concentrations, activation was low in all the above areas. In this aspect, it would be of great interest to determine the activation pattern of ERα-containing neurons during the follicular phase which may reveal different cellular activation patterns.

Kisspeptin cell activation during the follicular phase of controls.

In the ewe, there are contradictory results concerning which of the two main populations of kisspeptin cells (the ARC or the mPOA) are involved in positive estradiol feedback (11, 15). This discrepancy between results may be due to the use of ovariectomized or intact animals with high doses of supplementary estradiol that could produce different c-Fos activation patterns than are normally seen in intact animals with physiological estradiol concentrations. In the present study, the LH surge of intact ewes was accompanied by an intense activation of kisspeptin neurons in both the ARC and mPOA. Regarding the ARC, all regions (rostral, middle and caudal) contributed equally to the increase. This, together with the observation that estradiol acts in the MBH to induce the GnRH/LH surge in the ewe (3-4), indicates that ARC kisspeptin cells are involved in estradiol positive feedback to stimulate GnRH/LH surges. However, as the estradiol signal initiating the GnRH surge begins well in advance of the surge itself (36), we cannot conclude that ARC kisspeptin cells are solely responsible for the feedback effects of estradiol, but are most likely only involved in the surge secretion mechanism. Nevertheless, we cannot ignore the possibility that activation in ARC kisspeptin neurons may have occurred at other times than those we examined in the present study; or the 11% of ARC kisspeptin cells that were activated prior to the surge were sufficient to transmit the positive estradiol signal to GnRH neurons.

Turning to the mPOA, we observed a gradual increase in kisspeptin activation during the follicular phase with maximum activation during the surge. Two recent studies also report an increase in c-Fos activity of the mPOA kisspeptin cells at the time of the preovulatory LH surge (15) and an increase in kisspeptin mRNA in the late follicular phase (11). However, as mentioned above, in the ewe, there is evidence to

show that estradiol acts in the MBH, not the POA, to induce the LH surge (3-4) so we speculate that mPOA kisspeptin neurons are activated secondarily (possibly via the ARC) during estradiol positive feedback.

Kisspeptin has also been implicated in estradiol negative feedback (9). In our study, kisspeptin activation remained low during the follicular phase except at the time of the LH surge. One way of interpreting these results is that a low level of estradiol inhibits the expression of kisspeptin, leading to reduced GnRH secretion, consistent with negative feedback regulation.

Cellular activation after LPS.

In the present study, LPS administration in the late follicular phase lowered estradiol plasma concentrations and activated specific brain areas. Interestingly, an increase in activation was observed in the ARC, VMN, mPOA, PVN and dBb as early as 3h after treatment, whereas estradiol decreased 8h after the administration of LPS. Our results concur with evidence suggesting that there are at least two mechanisms activated in the hypothalamus during LPS inhibition of the ovarian cycle; one involving disruption of GnRH/LH pulses and, therefore, reducing estradiol secretion; and the other, preventing the ability of the surge-generating mechanism to respond to the preovulatory estradiol increase (42). Collectively, these results suggest that LPS acts within the ARC, VMN, mPOA, PVN and dBb to activate cells that inhibit the positive feedback effects of estradiol and, therefore, block the GnRH/LH surge. A similar mechanism may exist for the inhibition of sexual behavior. The precise phenotype of activated cells remains to be elucidated, however, strong evidence from the rat suggests that LPS administration stimulates the synthesis of γ-aminobutyric acid (GABA; 43), and this activation could account for an important inhibiting mechanism for the disruption of the LH surge and/or sexual behavior after LPS.

Kisspeptin cell activation after LPS.

LPS markedly decreased the proportion of activated kisspeptin cells in the ARC and mPOA, and this was evident 12h after treatment, at a time when control animals

were exhibiting an LH surge. Our data provide evidence for the potential existence of multiple kisspeptin cell inhibiting pathways. Firstly, as LPS is known to attenuate GnRH/LH pulses (42) and lower estradiol concentrations (2; and the present study), as well as the fact that kisspeptin cells are regulated by steroids (9, 11, 14), it may be that the failure of kisspeptin cells to become activated reflects the lack of a sufficient estradiol signal. In this regard, both cortisol and progesterone suppress pulsatile GnRH/LH secretion (44-46) and were elevated after the administration of LPS. Secondly, there are numerous signaling pathways activated by LPS that may influence kisspeptin cells directly. Our data indicate that there are a large number of cells activated in the hypothalamus/POA and these could provide inhibitory inputs to kisspeptin cells. We also found an increase in CRFR Type 2 immunoreactivity in the lower part of the ARC and the ME immediately after LPS, presumably in response to an intense activation in the PVN, where CRF cells are located (47). In rats, CRF administration i.c.v. profoundly decreases kisspeptin and kisspeptin receptor mRNA levels in both the anteroventral periventricular nucleus (AVPV) and ARC (18). Whether kisspeptin cells contain CRFRs remains to be investigated in any species. However, the kisspeptin and CRFR Type 2 distributions overlap and, therefore, interactions between the two systems seem likely. Certainly, CRFR Type 2 appears to be involved in LPS induced disruption of the LH surge. Similarly, cortisol and progesterone have been implicated in the disruption of the positive feedback effect of estradiol to trigger an LH surge (37, 48-50). Progesterone achieves this by directly preventing the activation of estradiol-responsive cells in the hypothalamus/POA (37), and therefore, it is likely that this includes kisspeptin cells. By contrast, evidence for a hypothalamic effect in cortisol disrupting the surge mechanism is lacking. However, glucocorticoid receptors Type 2 are abundant in the ARC and POA of the ewe (51) where kisspeptin cells are located; co-localization between the two components merits specific investigation.

Conclusion

Our results extend current knowledge regarding the mechanisms that control sexual behavior and the GnRH/LH surge, as well as their disruption after acute immune/inflammatory stress. We report that the LH surge of intact ewes is

accompanied by an intense activation within the VMN, ARC and mPOA, as well as an activation of kisspeptin cells located in the ARC and mPOA. Taking into account that the GnRH surge mechanism consists of three phases (activation, transmission and surge secretion), our results indicate that these hypothalamic regions, as well as both major populations of kisspeptin cells, are involved only in the surge secretion phase. Therefore, the cells that become activated prior to the LH surge when estradiol concentrations are initially elevated remain to be phenotyped. The BNST was activated just before the expected onset of sexual behavior and based on current neuroanatomical data we hypothesize that this may reflect the transmission of pheromonal signals from the amygdala. By contrast, acute LPS treatment prevented the LH surge from occurring and kisspeptin cells were not activated. This was accompanied by cellular activation in specific hypothalamic regions and an increase in CRFR Type 2 immunoreactivity in the lower part of the ARC and the ME. Both these mechanisms appear to be involved in the stress-induced disruption of the LH surge. The phenotype of the activated cells in response to LPS merit specific investigation.

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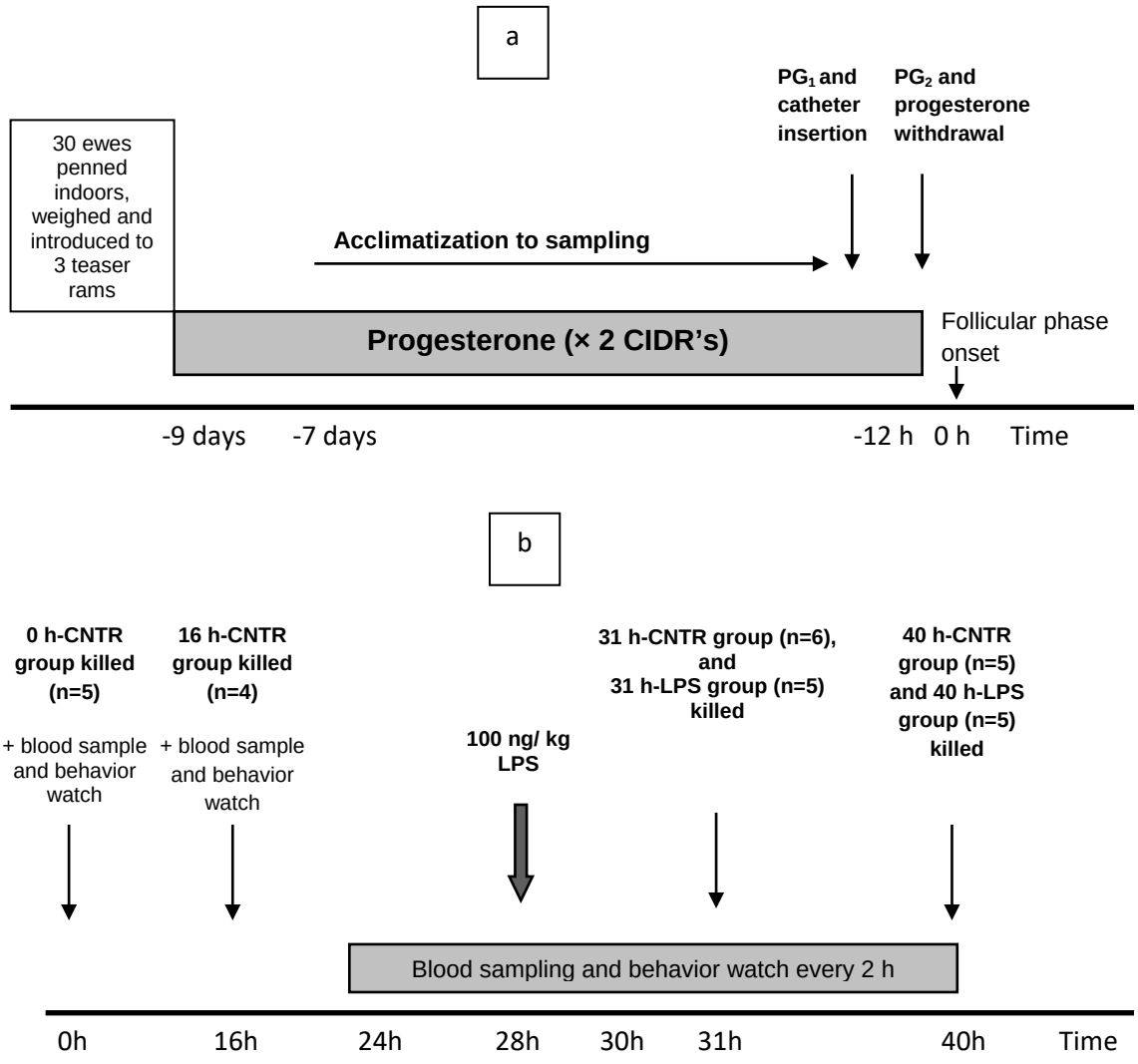

Fig.1 Diagram of the experimental protocol (a) before and (b) after the onset of the follicular phase. CNTR = control; LPS = E. coli lipopolysaccharide

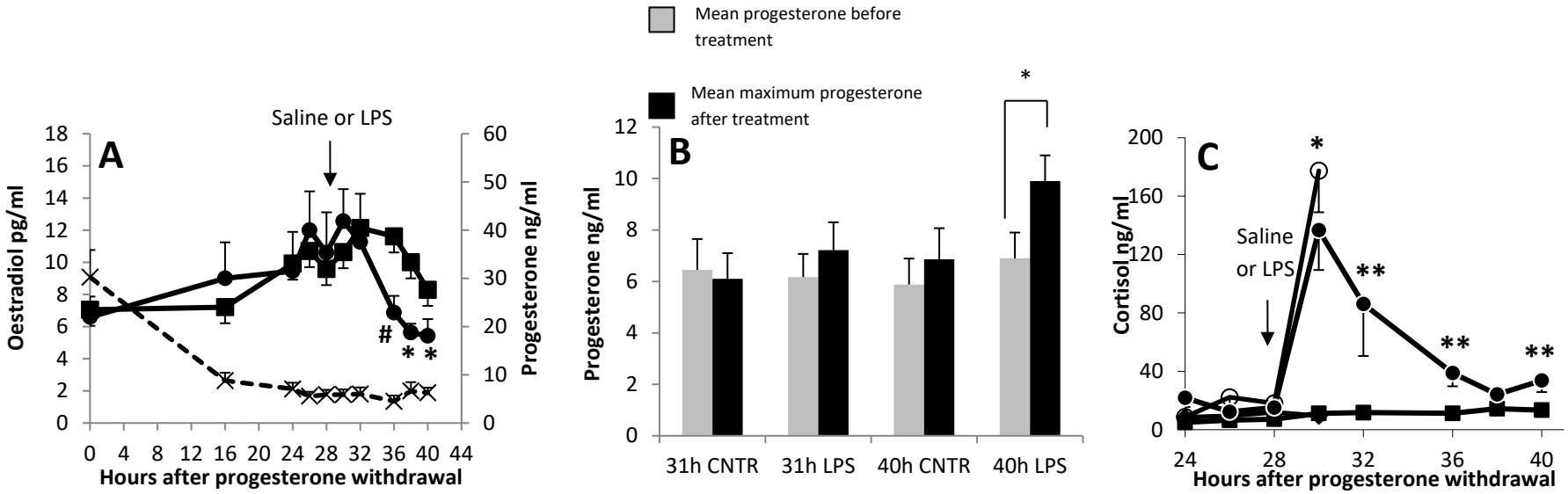

Fig. 2 A) Mean (±SEM) plasma estradiol concentrations in 40h control (■; n=5) and 40h LPS (●; n=4) groups. Mean (±SEM) plasma progesterone concentrations in 40h control animals (×; n=5) during the follicular phase are also shown for comparison with estradiol profiles. # Time at which 40h LPS group tended to differ from the 40h control group (P<0.07). *Time at which 40h LPS group differed from the 40h control group (P<0.02). B) Mean (±SEM) plasma progesterone concentrations at 28h after PW (before treatment; grey bars) and two mean (±SEM) consecutive maximum concentrations recorded after treatment (black bars), in the 31h control (31h CNTR; n=5), 31h LPS (n=4), 40h control (40h CNTR; n=5), and 40h LPS (n=4) groups. Due to the considerable between-animal variation, a within-group comparison was made. The differences between concentrations within an animal are linked by the line (* P<0.05). C) Mean (±SEM) cortisol concentrations in 31h control (♦; n=6), 31h LPS (o; n=4), 40h control (■; n=5) and 40h LPS (●; n=4) groups. * Time at which cortisol values from all treated groups were differed from the control groups (P<0.003). ** Time at which 40h LPS group values differed from the control group (P<0.05). The arrows indicate time of treatment. Some error bars are within the data symbols.

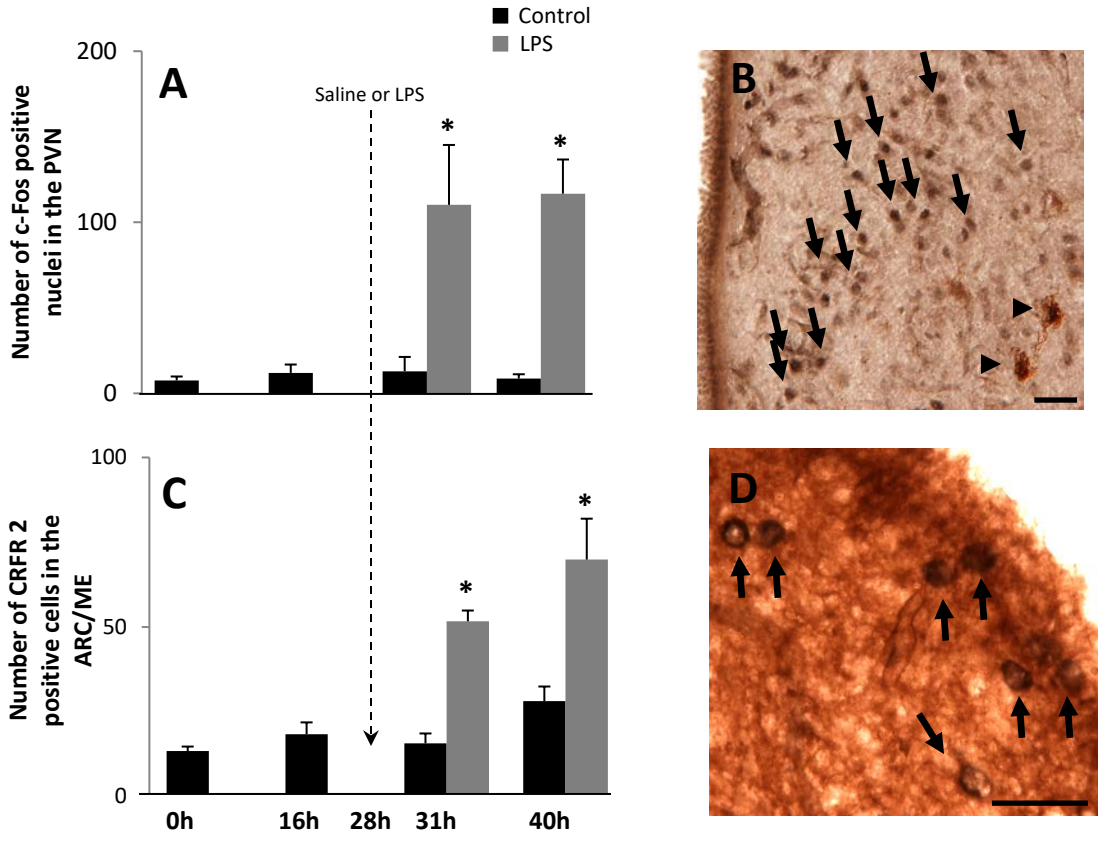

Fig. 3 A) Mean (±SEM) number of c-Fos positive nuclei in the PVN of control ewes, B) photomicrographs of the PVN stained for c-Fos (black arrows) and kisspeptin (*black arrow heads*) in an LPS treated ewe. *Scale bar* 50 μm. C) Mean (±SEM) number of CRFR type 2-positive cells in the lower part of the ARC and ME, D) Immunohistochemically identified CRFR type 2-positive cells (black *arrows*) in the lower part of the ARC and ME (Scale bar: 20 μm). Animals are grouped according to time after PW i.e. control ewes at 0h, 16h, 31h and 40h (n=4-5 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars). Treatment with saline or LPS was at 28h after PW (indicated by dashed arrow). * $P<0.05$ compared to control groups.

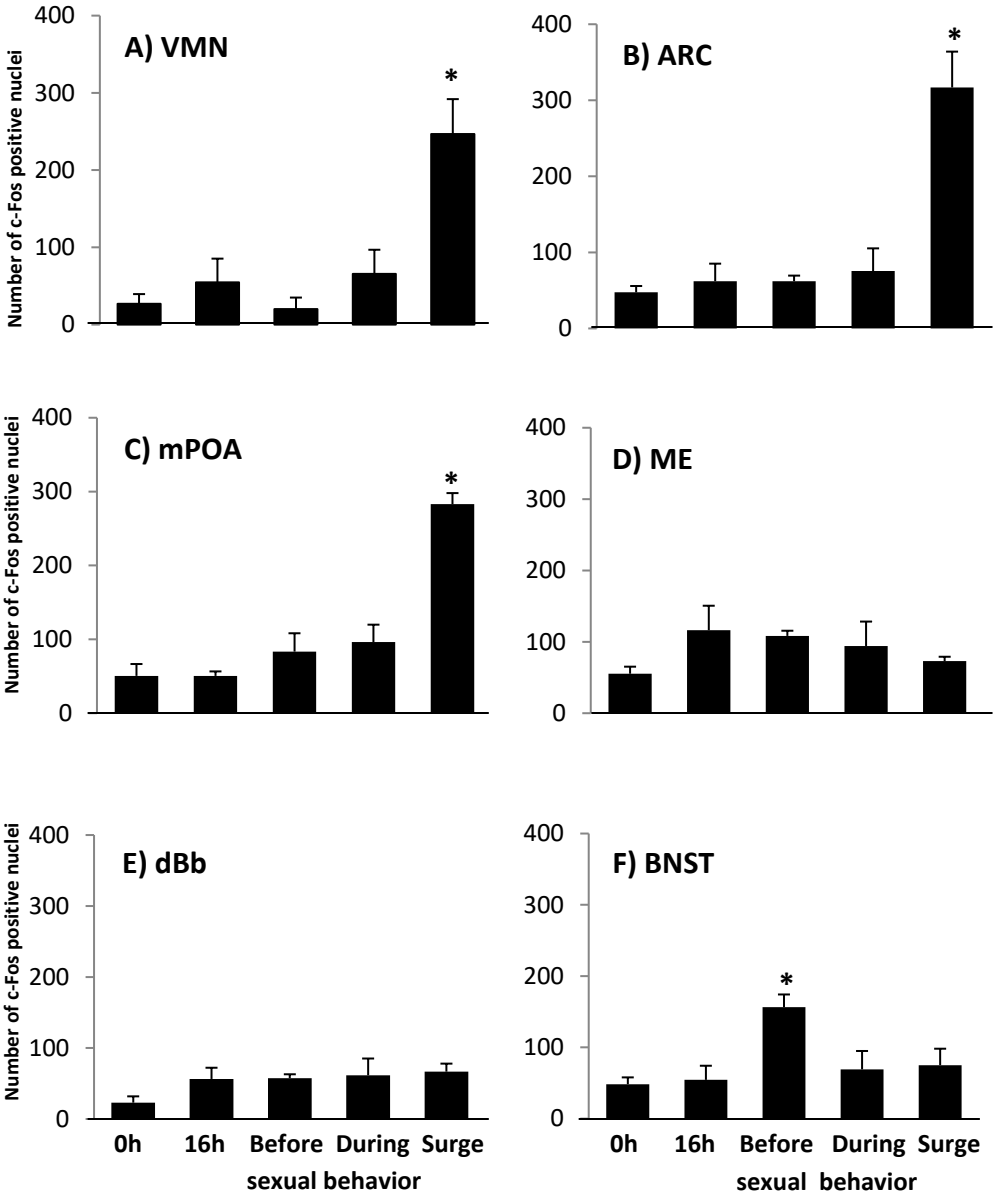

Fig. 2 Mean (±SEM) number of c-Fos positive nuclei in the A) VMN, B) ARC, C) mPOA, D) ME, E) dBb and F) BNST, at different stages in the follicular phase of control ewes. Animals were grouped according to time as well as hormonal and behavioural status; i.e., grouped into those killed at 0h and 16h after PW (n=4-5), those killed at 31h or 40h after PW but before the onset of sexual behaviour and the LH surge (Before sexual behaviour, n=3), those killed at 31h or 40h after PW, during sexual behaviour but before the LH surge onset (During sexual behavior, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3).***** *P*<0.05 compared to all other stages in the follicular phase.

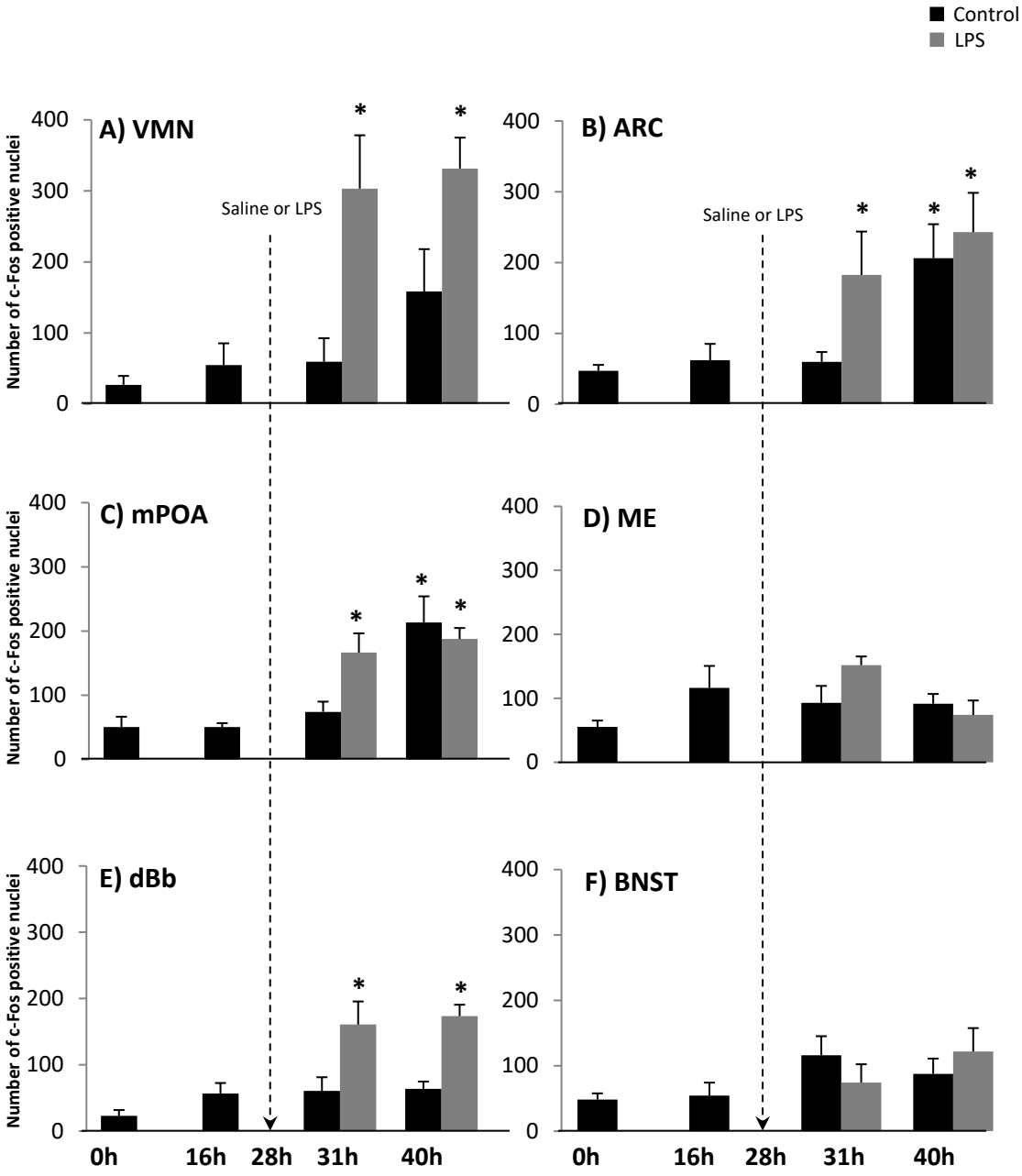

Fig. 5 Mean (±SEM) number of c-Fos positive nuclei in the A) VMN, B) ARC, C) mPOA, D) ME, E) dBb and F) BNST, at different times during the follicular phase of control and treated ewes. Animals are grouped according to time after PW i.e. control ewes at 0h, 16h, 31h and 40h (n=4-5 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars). Treatment with saline or LPS was at 28h after PW (indicated by dashed arrow). Fig 5A:***** *P*<0.05 compared to controls. Fig. 5B and 5C: * *P*<0.05 compared to 0h, 16h, 31h controls. Fig 5E: * *P*<0.05 compared to control and insulin subgroups combined.

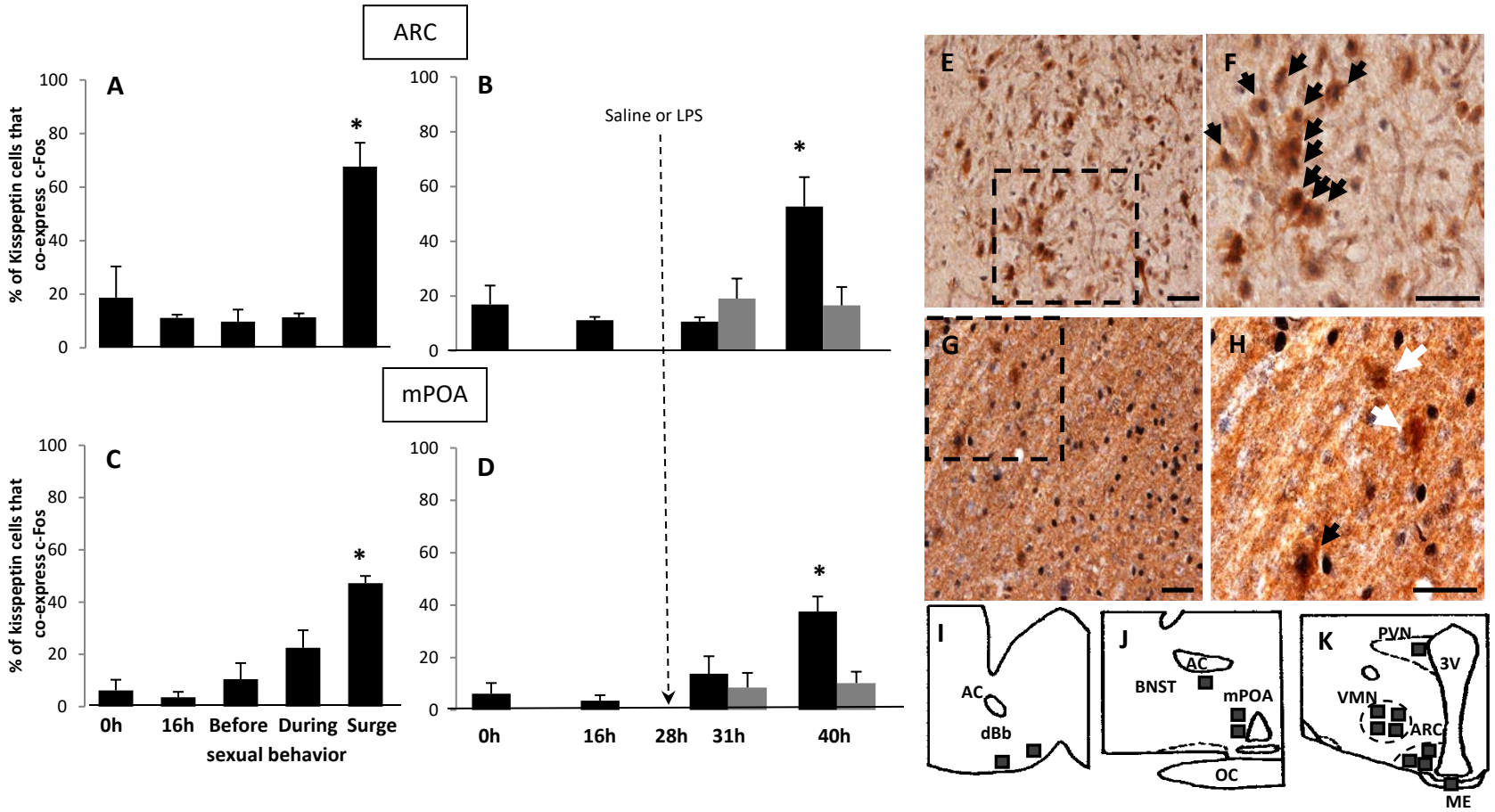

Fig. 3 Mean (±SEM) % number kisspeptin cells that co-express c-Fos in the ARC A) and B) and mPOA C) and D) during the follicular phase of intact ewes. Animals in A and C are grouped according to time as well as hormonal and sexual behavioural status; see legend for Fig 2. In B and D animals are grouped according to time after PW i.e. control ewes at 0h, 16h, 31h and 40h (n=4-5 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars Treatment with LPS was at 28h after progesterone withdrawal (indicated by dashed arrow). *P<0.05 compared to all other groups within each panel. E-H) Photomicrographs of the ARC nucleus that were dual-labelled for kisspeptin cells and their co-expression with c-Fos in control animals during the LH surge (E and F) as well as 12 h after LPS treatment in the late follicular phase (G and H). The *right panels* in each section are the higher magnifications (20 μm) of the *boxed areas* shown in the *left panels* (50 μm). *Black arrows* indicate examples of dual-labelled cells, and *white arrows* indicate single-labelled kisspeptin-

positive cells. I-K) Schematic diagrams illustrating the photographs (grey box) taken and considered for analysis in each area. AC: anterior commissure, dBb: diagonal band of broca, BNST: bed nucleus of the stria terminalis, mPOA: medial preoptic area, OC: optic chiasm, PVN: paraventricular nucleus, VMN: ventromedial nucleus, ARC: arcuate nucleus, ME: median eminence, 3V: third ventricle.

Table 1 Time of the onset of the LH surge, pre-copulatory behavior and estrus (hours after PW) of individual ewes treated with saline or LPS at 28h after PW. Ewes were killed at 0h, 16h, 31h (31h control and 31h LPS groups) or 40h (40h control and 40h LPS groups) after PW. There was no sexual behaviour or LH surge recorded in control ewes killed at 0h or 16h.

	Near Ram	Being nosed	Being nudged	Mounted	LH surge
31h CNTR					
Sheep B					
Sheep C					
Sheep D	25	27	27		
Sheep E	26	26			
Sheep F					
Sheep P	25	25			
31h LPS					
Sheep Z					
Sheep Δ	27	27	29	29	
Sheep Я					
Sheep Ω					
40h CNTR					
Sheep H	38	38	38		
Sheep I	40	36	40	40	
Sheep K	24	24	28	32	38
Sheep L	22	22	34	34	38
Sheep M	28	30	30	30	34
40h LPS					
Sheep T	26	26			
Sheep θ					
Sheep®					
Sheep Ξ	34	34	34	34	