UNIVERSITY OF LIVERPOOL

School of Veterinary Clinical Science

Hormone, Behaviour and Neuropeptide Profiles of Normal and Stressed Ewes

Thesis submitted in accordance with the requirements of the

University of Liverpool

for the degree of

Doctor of Philosophy

by

Chrysanthi Fergani

September, 2011

Declaration

Unless otherwise acknowledged, this thesis is all my own work, undertaken in the Department of Veterinary Clinical Sciences and Animal Husbandry, University of Liverpool, Veterinary Field Station, Leahurst, U.K., and under the supervision of Professor Hilary Dobson, Dr. Robert Frank Smith and Dr. Lucy Pickavance. No part of this thesis, in any form has been submitted to any other university or for any other degree.

Chrysanthi Fergani

Acknowledgements

I would like to thank my supervisor Professor Hilary Dobson for being a true inspiration throughout the 5 years I have worked with her. Thank you for trusting me and for giving me the opportunity to do a PhD, for your endless encouragement, this kept me trying harder. Thank you for teaching me how to stand on my own two feet, to take responsibility for my work and think for myself. Every word you have said has been engraved in my brain! You have been a wonderful role model and please know that your way of teaching and scientific ethos will follow me throughout my career, whatever that may be. Above all thank you for caring.

I would also like to thank Dr. Rob Smith, for making time to provide valuable input, come up with ideas and help with statistics. Your comments and suggestions are greatly appreciated and always made a big difference to my understanding of things. Thank you to Lucy Pickavance for allowing me unlimited access to the lab and making time to suggest ideas.

A great big thank you to Jean Routly, for her caring nature and inventive mind, which have gotten me out of many sticky situations! Your contribution was priceless and it was a pleasure to work with you.

I would also like to thank everyone else who helped with the animal work: Dr. Michael Morris, Dave Jones, Hillary Pursell, Asimakis Giousas, Hayley Gough, Hadi Darbari, Salvatore Canu. This was a team effort and a great experience.

I would like to thank my 'UK family': Kealan, Mary, Maya, Ed and Matthew. You are all a part of me and therefore this PhD. I don't even want to think about what it would be like not seeing you every day. Ed and Matthew: I am truly going to miss the little back yard with the brewing pot.

I would like to thank my parents, for knowing that I should be a researcher, and pushing me towards that direction. Thank you for telling me that I have made you proud, it has made me very happy and worth every effort I have made. Thank you for bringing me up to be crazy enough to want to do a PhD. Mother: you are the inexhaustible source of my strength. I am very lucky to be your daughter. Every word in this thesis I owe to you.

Abstract

Hormone, Behaviour and Neuropeptide Profiles of Normal and Stressed Ewes

Chrysanthi Fergani

September, 2011

The aim of the present study was to investigate the hormone, sexual behaviour and neuropeptide profiles of follicular phase ewes and examine alterations after the application of acute stressors. In study 1, follicular phases of intact ewes were synchronised with progesterone vaginal pessaries. Ewes then received saline vehicle, insulin (4 iu/kg) or endotoxin (LPS; 100ng/kg) at 28h after progesterone withdrawal (PW; time zero). In study 2, this protocol was repeated, but animals were killed at 0h, 16h, 31h and 40h after PW and brain tissue retrieved. In study 1, there was a delay of 17.6h and 7.2h (P<0.05), respectively, in half the insulin-treated animals ('insulin-delayed') but not in the other half; and a delay of 22.5h and 20.7h (P<0.001), respectively, in all LPS-treated animals. Plasma oestradiol concentrations decreased after both stressors (P<0.001) and cortisol increased in all groups (P<0.05); whereas progesterone increased in the insulin-delayed and LPS groups only (P<0.05). In study 2, immunohistochemistry was used to examine transcriptional activation (co-expression with c-Fos) of various neuropeptides in the hypothalamus and preoptic area. In control ewes, the maximum percentage of dynorphin cells co-localising c-Fos (i.e., activated) was observed at 31h after PW (52%; P<0.05), whereas maximum activated kisspeptin and neurokinin B cells occurred at 40h after PW (49 and 42%, respectively; P<0.05). The percentage of activated dopamine cells decreased before the onset of sexual behaviour (from 70 to 26%; P<0.05) whereas β-endorphin activation was lower during the LH surge (from 41 to 10%; P<0.05). In contrast, neuropeptide Y and somatostatin activation was higher during the surge (from 21 to 36%; P<0.08; and from 14 and 9% to 47 and 73%, respectively; P<0.05). However, LPS decreased the percentage of activated dynorphin cells (to 11%; P<0.05) and kisspeptin cells (to 22%; P<0.05). On the contrary, insulin decreased the percentage of activated dynorphin cells (to 27%) in two of the insulin-treated animals (insulin-responders) but not in the other two; whereas the percentage of activated kisspeptin cells increased in all insulin-treated animals (52%; P<0.05). Neurokinin B was not altered by either treatment. Furthermore, insulin increased the percentage of activated β-endorphin, neuropeptide Y and somatostatin cells in the ARC (to 71, 72 and 63%, respectively, P<0.05) but LPS did not have the same effect. In the VMN, activation of somatostatin cells was greater in all LPS treated animals (from 8 to 27%; P<0.05) but only in two of the insulin-treated animals (to 55 and 76%; insulin-responders) but not in the other two (to 5 and 6%; insulin-non-responders). These results indicate that there is a specific hormonal, behavioural and neuropeptide pattern during the follicular phase of intact ewes and this is disturbed by acute LPS or insulin administration in the late follicular phase, leading to the disruption of the LH surge.

Table of Contents

List of Abbreviations

ACTH	adrenocorticotrophic hormone
AHA	anterior hypothalamic area
ARC	arcuate nucleus
AVP	arginine vasopressin
BNST	bed nucleus of the stria terminalis
CAG	chrome alum gel
cm	centimetres
CRF	corticotrophin releasing factor
dBB	diagonal band of broca
DHA	dorsal hypothalamic area
e.g.	for example
EIA	enzyme immunoassay
ER (α or β)	oestradiol receptor (alpha or beta)
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GLM	genaral linear model
gr	gram
GnRH	gonadotrophin releasing hormone
h	hour(s)
H-P-A	hypothalamic-pituitary-adrenal
H-P-O	hypothalamic-pituitary-ovarian
IU	international unit(s)
i.c.v	intracerebroventricular
i.v.	intravenous
kg	kilogram(s)
KNDy	kisspeptin-neurokinin B-dynorphin
LH	luteinising hormone
LHA	lateral hypothalamic area
LS	lateral septum
MBH	mediobasal hypothalamus
ME	median eminence
mg	milligram(s)
min	minutes
ml	milliliter(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger ribonucleic acid

ng	nanogram(s)
OR (μ, δ or κ)	opioids receptor (mu, delta or kappa)
OVLT	organum vasculosum of lamina terminalis
OVX	ovariectomised
PB	phosphate buffer
PBS	phosphate buffered saline
PeVA	periventricular area
POA	preoptic area
mPOA	medial preoptic area
POMC	pro-opiomelanocortin
PR	progesterone receptor
PVN	paraventricular nucleus
RIA	radioimmunoassay
SEM	standard error of the mean
SST	somatostatin
T	time
TH	tyrosine hydroxylase
VMN	ventromedial nucleus
3V	third ventricle
μg	microgram(s)
μm	micrometers
<	less than
>	more than
%	percent
^{0}C	degrees celsius

Chapter 1

Literature Review

Introduction

For the successful production of offspring in all mammals, it is essential for the ovum and sperm to be in contact at the appropriate time. This is achieved by the precise coordination of complex physiological and neuroendocrinological events involving the hypothalamic-pituitary-ovarian axis (H-P-O axis), which controls, in close synchrony, behaviour changes during oestrus as well as the preovulatory surge release of the gonadotrophin hormones. Interestingly, both reproduction and stress, (which is a disruptor of these events), are controlled by similar nuclei and neurotransmitters in the brain (Dobson *et al.* 2003). It is therefore useful to study pathological and physiological mechanisms in comparison.

The GnRH network

GnRH and the control of reproduction

The final common pathway controlling reproductive function in vertebrates are the gonadotrophin releasing hormone (GnRH) neurones and their projections to the median eminence (ME), the site of peptide release into the pituitary portal system (Herbison 1998). GnRH is released at the ME into the capillaries of the pituitary-portal plexus, and transported to gonadotrophes of the anterior pituitary to stimulate production and secretion of LH (luteinising hormone) and FSH (follicle stimulating hormone). From then on, these two hormones enter the peripheral circulation in order to reach the ovary and induce folliculogenesis and steroidogenesis (Skinner *et al.* 1995, Goodman, 1996). The activity of GnRH neurones, are in turn, controlled by the positive and negative feedback actions of the two ovarian steroids; oestradiol and progesterone (Moenter *et al.* 1991). GnRH release is also regulated by several molecules, including neurotransmitters and glial cell factors (Herbison 1998). There is a theory which supports that mammals have two forms of GnRH, GnRH I, which regulates gonadotrophin secretion, and GnRH II, which appears to be a neuromodulator and plays a role in sexual behaviour (Pawson *et al*. 2003) but this, has not been confirmed to date.

GnRH secretion mode

During the ovine oestrous cycle, GnRH secretion can be divided into the following components: 1) tonic or pulsatile secretion, where groups of GnRH neurones are activated synchronously and the hormone is released in boluses (pulses) stimulating gonadal growth and steroidogenesis 2) the preovulatory GnRH surge, which consists of a massive and sustained release causing ovulation and luteinising the follicle in to a corpus luteum (CL), and possibly 3) an unknown mode of secretion responsible for the onset and/or maintenance of oestrous behaviour (Bowen *et al.* 1998). It is now well established that the GnRH surge is induced by the positive feedback action of oestradiol, whereas pulsatile GnRH secretion is controlled by the negative feedback action of both oestradiol and progesterone (Goodman, 1996). There is tight coupling between the secretory bursts of GnRH and those of LH (Clarke *et al.* 1987, Bowen *et al.* 1998) with one pulse of LH corresponding from one pulse of GnRH. A sustained volley of high-frequency pulses of GnRH secretion is a fundamental step in the sequence of neuroendocrine events leading to ovulation during the breeding season of sheep (Barrell *et al.* 1992).

GnRH neurone distribution

Jansen *et al.* (1997) have identified the distribution of GnRH neurones in the sheep hypothalamus using neuronal tract-tracing compounds (Fig. 1). They report that the majority of GnRH neurones were found in the medial preoptic area (mPOA) at the level of the organum vasculosum of the lamina terminalis (OVLT), and these accounted for approximately 54% of the total GnRH neurone population. By contrast, GnRH neurones in the diagonal band of Broca/medial septal region (dBB/MS), anterior hypothalamic area/ lateral hypothalamic area (AHA/LHA) , and the arcuate nucleus (ARC) of the mediobasal hypothalamus (MBH) composed 15%, 22%, and 9% of the total GnRH population, respectively. GnRH cells that express c-Fos (a neuronal marker for activation) during the surge are not anatomically segregated but rather distributed heterogeneously among various brain regions (Jansen *et al.* 1997). The GnRH perikarya located in the mPOA are considered the most important final sites of regulation by neuronal populations located in the brain stem, the MBH and the mPOA and therefore for the generation of the GnRH surge (Dobson *et al.* 2003). In contrast, GnRH neurones

responsible for maintaining pulsatile LH secretion are speculated to reside in discrete regions, specifically the MBH (Jansen *et al.* 1997). Whether there is a distinct population of GnRH neurones for the control of sexual behaviour, or some of these neurones are able to control both events remains unknown.

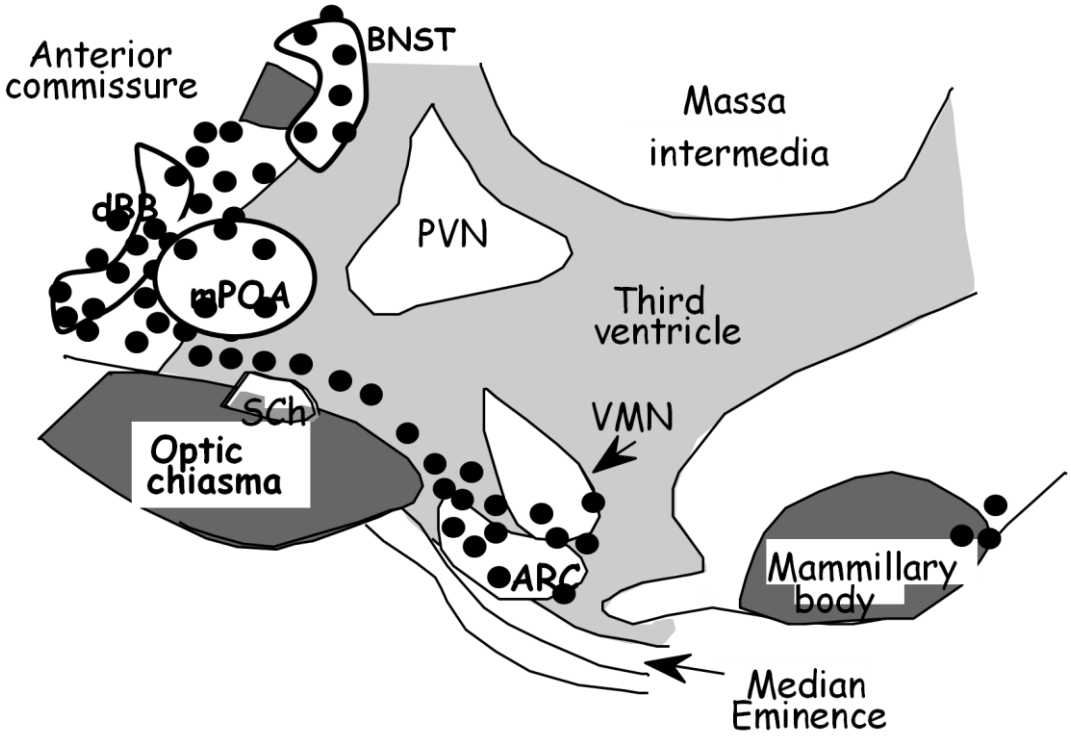

Fig. 1 Distribution of GnRH cell bodies in the ovine preoptic area and hypothalamus (Original from D. Blache).

Steroid control of reproduction

The ovarian steroid hormones oestradiol and progesterone are of central importance in the control of reproductive neuroendocrine function in female mammals. These hormones exert key regulatory actions on fertility by altering the activity of excitatory and inhibitory neural circuits that control the release of GnRH. Although it is known that both steroids exert their actions on GnRH release via nuclear receptors (Taylor *et al.* 2007), the mechanisms by which ovarian hormones modulate both tonic and surge modes of GnRH release, as well as oestrus behaviour remain unclear. Direct measurements of GnRH in hypophyseal portal blood have provided strong evidence that

both oestradiol and progesterone inhibit GnRH pulses, and they do so by different mechanisms. Oestradiol alone inhibits LH (Goodman and Karsch 1980) and GnRH (Evans *et al.* 1994) pulse amplitude, whereas progesterone by itself inhibits LH (Goodman and Karsch 1980)and GnRH (Skinner *et al.* 1998) pulse frequency, an action that is enhanced by oestradiol (Goodman *et al.* 1981). However, during the late follicular phase elevated oestradiol triggers the preovulatory GnRH surge at the end of the follicular phase and initiates sexual behaviour.

Steroids control the GnRH network indirectly

GnRH neurones constitute the final common pathway of a complex neuronal network responding to the circulating steroid hormone milieu to control ovulation and sexual behaviour (Karsch *et al.,* 1997, Caraty *et al.,* 2002). However, steroid hormone signals do not impinge directly on GnRH cells as they do not possess progesterone receptors (PR) or oestradiol receptors subtype α (ERα; Shivers *et al*., 1983, Herbison and Theodosis 1992, Lehman and Karsch, 1993, Sullivan *et al.* 1995, Scott *et al.* 2000, Skinner *et al*., 2001). GnRH neurones have been reported to contain oestrogen receptor β (ERβ; Skynner *et al.,* 1999; Hrabovszky *et al.,* 2001, Herbison *et al.* 2001, Jansen *et al.,* 2001, Skinner and Dufourny 2005) but their role is not fully elucidated, although considered minor (Scott *et al.* 2000, Orikasa and Sakuma 2003, Maeda *et al.,* 2010) because ERβ-knock out mice show normal fertility (Lubahn *et al.,* 1993; Krege *et al.,* 1998). Thus, it is generally accepted that cells in the brain that possess ERα are the main mediators of the feedback effects of steroid hormones which in turn secrete neurotransmitters to influence the GnRH network (Caraty *et al*. 1998, Herbison 1998, Smith and Jennes 2001, Herbison 2008). Ther phenotypes of cells that contain Erα and have been discovered to date are shown in Table 1. These cells are potential mediators of steroid feedback.

Progesterone negative feedback

In sheep, this action of progesterone appears to be mediated by the classical nuclear PR (Skinner *et al.* 1998), but as mentioned above, PR are generally not found in GnRH neurones in ewes and other species (Skinner *et al.* 2001). Thus, other systems most likely transmit the actions of progesterone to GnRH neurones. There is now general agreement that endogenous opioid peptides (EOP) are one of the major systems that

mediate progesterone negative feedback in sheep (Goodman *et. al.* 2004, Faradori *et al.* 2005) rats (Kalra 1993). Although there are similarities in the EOP mediation of progesterone negative feedback in sheep, rats, and primates,there is one difference in this action of progesterone among these species. In rats (Goodman, 1978), the negative feedback action of progesterone requires the presence of oestradiol; whereas in sheep, progesterone alone inhibits tonic LH secretion in OVX animals (Karsch *et al.,* 1997). OVX in ewes increases dynorphin concentrations in CSF collected from the third ventricle and preprodynorphin (PPD) mRNA in subpopulations of dynorphin neurones. Therefore it is hypothesised that dynorphin mediates progesterone negative feedback (Faradori *et al*. 2005).

Oestradiol negative feedback

During the follicular phase oestradiol influences GnRH secretion with both negative and positive feedback mechanisms which are believed to be independent (Dobson etal. 2003, Scott *et al.* 2000). It has been suggested that LH pulse frequency varies depending on the concentration of oestradiol (i.e. different parts of the brain are activated for the two feedback mechanisms depending on the 'dose' of oestradiol; Thiery and Martin 1997). It is also believed that the neuronal cell populations that are responsible for the negative and positive feedback actions of oestradiol are, as mentioned above, different (Caraty *et al.* 1998). Many of the actions of oestradiol in the adult brain are thought to result from intracellular binding to nuclear ER (Blaustein and Olster 1989). Oestradiol negative feedback action on LH secretion is rapid, so classic genetic actions of oestradiol through nuclear receptors might not be involved in the feedback mechanism. In this regard, a unique non-classical action of ER was first proposed by the group led by Levine (McDevitt *et al.,* 2008). In addition, it is possible that membrane ER, such as GPR30, are involved in the rapid suppression of GnRH/ LH secretion by oestradiol. Actually, GPR30, a membrane receptor of oestradiol, is also expressed in primate GnRH neurones and might be involved in mediating rapid oestradiol action (Noel *et al.,* 2009). Moenter's group (Sun *et al.,* 2010) has proposed that multiple mechanisms including both direct actions of oestradiol on GnRH neurones via GPR30 and ERβ and indirect actions via ERα in neurones afferent to the GnRH neurone must play a critical role in oestradiol feedback regulation of LH secretion.

Table 1. Neuronal cells that contain ERα in the ovine hypothalamus preoptic area and brainstem.

Neurotransmitter	Nucleus	Percentage of cells that contain ERα	Reference
Tyrosine Hydroxylase (Dopamine)	ARC	<10% of total ER-containing cells	Batailler *et al.,* 1992
		3-5%	Lehman and Karsch, 1993
		15%	Skinner and Herbison, 1997
	POA	0%	Lehman and Karsch, 1993
		13-18%	Skinner and Herbison, 1997
	AHA	3-5%	Lehman and Karsch, 1993
		25%	Skinner and Herbison, 1997
β-endorphin	ARC	15-20%	Lehman and Karsch, 1993
GABA	POA	44%	Herbison *et al.,* 1993
Neuropeptide Y	ARC	3%	Skinner and Herbison, 1997
Somatostatin	VMN	35%	Herbison, 1995
	ARC	13%	Scanlan *et al.,* 2003
DβH (Noradrenaline)	VLM	20-70%	Simonian *et al.,* 1998
	NST	8-25%	Scott *et al.,* 1997
Kisspeptin	POA	50%	Franceschini *et al.,* 2006
	ARC	93%	Franceschini *et al.,* 2006
Glutamate	ARC/VMN	52-61%	Pompolo *et al.,* 2003
Galanin	Hypothalamus and POA	50%	Tourlet *et al.,* 2005
GnRH	POA	0%	Herbison *et al.,* 1993
		0%	Lehman and Karsch, 1993

Oestradiol positive feedback

In ruminants, oestradiol is considered as the primary "trigger" for the behavioural and the endocrine changes observed during the follicular phase (Fabre-Nys and Gelez 2007, Fabre-Nys *et al.*1994). Physiologically, in the ewe, as in many other species, oestradiol secretion from the ovarian dominant follicle(s) increases during the late follicular phase of the oestrous cycle. This increase exerts a 'positive feedback' action in central nervous system structures, to generate the LH surge and ovulation (Clarke *et al.* 1987, Caraty *et al.* 1989, Moenter *et al.* 1990, Goodman 1996). This is achieved both with an increase in

pituitary sensitivity (acting directly upon gonadotrophes to induce synthesis of GnRH receptors; Evans *et al.* 1997) together with an increase in GnRH secretion from the hypothalamus (Clarke and Cummins 1984). Interestingly, Ben Said *et al.* 2007 have recently reported that in two breeds of OVX ewes, the oestradiol concentration and duration of presence threshold for the induction of oestrus behaviour is different than that for the induction of the LH surge. Therefore, the activation of some oestradiol-responsive neurones for few hours in the MBH probably corresponds to a necessary early step, sufficient to trigger off a chain of events leading to the induction of the preovulatory surge and sexual behaviour

Neurotransmitters

In an effort to determine which one of these sites is responsible for the generation of the GnRH surge, using an ovariectomised ewe model, Caraty *et al.* (1998), suggested that the primary site of oestradiol positive action for GnRH surge release is the the MBH. Furthermore, the implantation of a small quantity of steroids in specific brain areas has led to the identification of the same area as the critical site for oestradiol and progesterone action on female sexual behaviour (Blache *et al*. 1991). The synchronous occurrence of the GnRH surge onset and sexual behaviour onset, as well as the fact that oestradiol is the triggering signal for both (Fabre-Nys and Martin 1991), by acting primarily on the VMN, raises the question of what neuronal regulatory mechanisms control each of these events separately. The neurotransmitters that are expressed in these ER-containing cells, and transmit the oestradiol signal to the GnRH network, are not yet fully determined. Furthermore, changes observed in neurotransmitter signaling account for both the GnRH surge and oestrous behaviour and complicate the efforts to fully elucidate reproductive mechanisms.

The regulation of hypothalamic GnRH secretion in the hypothalamus is provided by both stimulatory and inhibitory neurotransmitters/or neurohormones. As oestradiol-receptive neurones have been identified to project to the immediate vicinity of the GnRH cell bodies as well as the ME it is possible that ER-expressing neuronal populations influence GnRH neurones at both terminal and/or cell body levels in a direct or indirect manner. The changes that are reported around the time of the preovulatory GnRH surge

concern either the surge itself or the induction of oestrous behaviour and a distinction between the two cannot be made in physiological situations.

A series of neuronal systems converge on the GnRH cells to determine their output. Some of these are discussed below.

Kisspeptin

The G protein-coupled receptor GPR54, and its peptide ligand kisspeptin have recently been found in the brain and appear to play a key signalling role in the regulation of reproductive function in animal and human models (Pompolo *et al.* 2006). The *Kiss 1* gene encodes a large (132–145 amino acids) precursor that contributes to a family of smaller peptides, ranging from 10–54 amino acids, which act via GPR54 to stimulate GnRH and LH release in rodents (Navarro *et al.* 2005), sheep (Messager *et al.* 2005), monkeys (Plant *et al.* 2006), and humans (Dhillo *et al.* 2005). The biologically active site has been mapped to a 10 amino acid sequence at the C terminal of metastin (kisspeptin 10; Franceschini *et al.* 2006). While GPR54 is expressed in numerous tissues associated with reproductive function, there is accumulating evidence that kisspeptin acts predominantly at the level of the central nervous system to regulate GnRH secretion (Messager *et al.* 2005). Furthermore, a stimulatory action on GnRH secretion was provided by studies showing that icv-administered kisspeptin increased GnRH levels in the cerebrospinal fluid within the third ventricle (3V) of the sheep brain (Messager *et al.* 2005). GPR54 is expressed by GnRH neurones and is directly activated by kisspeptin to stimulate GnRH release in both rats and sheep (Irwig *et al.* 2004, Messager *et al.* 2005). Franceschini *et al.* (2006) have raised a highly specific antiserum against kisspeptin 10 and determined the anatomical distribution of kisspeptin-immunoreactive cells in the hypothalamus of the ewe. Kisspeptin immunoreactive cells were abundant in the caudal ARC, the dorsomedial nucleus and the mPOA, while only a few cells were observed in the VMN. A small number of scattered kisspeptin10-immunoreactive cells were also detected alongside the walls of the third ventricle throughout its entire rostro-caudal extent, including within the PVN. The most striking accumulation of kisspeptin10-immunoreactive cells was observed in the caudal ARC. Numerous varicose fibers were also seen running parallel to the walls of the third ventricle, some of which extending up to the PVN and some to the ME. These findings are consistent with the recent

immunofluorescent analysis of Pompolo *et al* (2006) with the difference that they report the existence of a distinct population of kisspeptin neurones in the periventricular nucleus (PeVN), as has been proven in rodents (Seminara, 2005). The pattern of distribution of kisspeptin immunoreactivity in the hypothalamus suggests a role for kisspeptin in the oestrogen-dependent regulation of GnRH and LH secretion in the ewe (Smith *et al*. 2005, Franceschini *et al.* 2006). In view of the increased association of kisspeptin10 fibers with blood capillaries in the ME, as well as the fact that GPR54 is expressed in the pituitary gland as indicated by PCR studies (Muir *et al.* 2001), the possibility that kisspeptin also act directly at the level of the pituitary gonadotrophes to potentiate LH secretion must also be taken into account. Furthermore Pompolo *et al*. (2006) report that GnRH and kisspeptin colocalise within cells of the POA and GnRH neurosecretory terminals of the ME and suggest that the two peptides might be cosecreted into the hypophyseal portal blood to act on the pituitary gland. However, the stimulatory effects in the above organ appear to be minor, as shown in rats (Navarro *et al.* 2005). In the same species, previous immunohistochemical studies failed to detect any kisspeptin immunoreactive cell bodies in the POA or AHA (Brailoiu *et al.* 2005, Kinoshita *et al.* 2005). In this species kisspeptin immunoreactive neurones were also detected in the PeVN and the well-defined sexually dimorphic nucleus called the anteroventral paraventricular nucleus (AVPV; Seminara, 2005). This difference may be due to species, but also due to differences in the sensitivity of detection methods. Within the caudal ARC of the ewe, nearly all kisspeptin immunoreactive cells co-express ERα compared with approximately half in the POA, indicating that there may be two different populations of kisspeptin neurones in this area. In rats 40–60%of kisspeptin neurones in the rostral PeVN of the 3V express ERα and PR (Clarkson *et al.* 2008), but similar results have not been reported in sheep. Pompolo *et al.* (2006) compared the number of immunoreactive kisspeptin neurones of ewes after ovariectomy and report an increase in the former cell type. This increase was observed on the ARC and not in the PeVN or POA kisspeptin population. This is inconsistent with findings in the rat where in the ARC kisspeptin mRNA expression increased after ovariectomy and decreased with oestradiol treatment and the reversed occurred in the PeVN and preoptic region (Smith *et al*. 2005). Therefore it can be assumed that these two kisspeptin cell populations represent major relays in the negative and positive feedback effects of oestrogen on

GnRH neurones, respectively (Dungan *et.al* 2006). Cells of the ARC are rapidly activated by oestradiol in the ewe, as indicated by c-Fos appearance within 1 h (Clarke *et al.* 2001). Furthermore, oestradiol-responsive cells of the ARC and VMH project to the BNST and POA (Pompolo *et al.* 2003, Pompolo *et al.* 2001), so there is a possibility that this pathway involves kisspeptin cells. Using GPR54- and Kiss1-null mouse models Clarkson *et al.* (2008) evaluated whether kisspeptin and/or GPR54 were essential for GnRH neurone activation and the LH surge. Whereas wild-type littermates all exhibited LH surges and c-Fos in 50% of their GnRH neurones, none of the mutant mice from either line showed an LH surge or any GnRH neurones with c-Fos. These observations provide the first evidence that kisspeptin–GPR54 signaling is essential for GnRH neurone activation that initiates ovulation. At present, the rodent rostral periventricular area of the 3V kisspeptin neurones are the only ones that meet all of the criteria necessary to be considered a key neural component of the positive feedback pathway: (1) They are sexually dimorphic favouring females in mice (Clarkson and Herbison 2006), (2) They express ERα and PR, (3) They project to GnRH neurones (Clarkson and Herbison, 2006), (4) They are activated by oestrogen positive feedback, and (5) GnRH neurones express GPR54 and are activated by kisspeptin (Irwig et al., 2004). This broadens considerably the potential roles and therapeutic possibilities for kisspeptin and GPR54 in fertility regulation (Clarkson *et al.* 2008). Contrasting results have been reported in sheep, where an increase in kisspeptin mRNA levels is observed in a subpopulation of ARC neurones just before the LH surge in ewes (Estrada *et al.* 2006) suggesting that this neuropeptide is most important in the positive feedback actions of oestradiol in this species. As mentioned before ovariectomy of sheep increases kisspeptin gene expression in the ARC, so these neurones may also play a role in steroid negative feedback (Smith *et al.* 2007). Nonetheless, it is now generally accepted that kisspeptin is tha major link between gonadal steroids and GnRH neurones (Carary *et al.,* 2010). Recent studies have shown that nearly all kisspeptin cells in the ARC (specifically the middle and caudal aspects), but not the POA, co-localise two other neuropeptides that are important in the control of GnRH secretion: neurokinin B and dynorphin (Goodman *et al.,* 2004, Goodman *et al.,* 2007, Topaloglu *et al.,* 2009, Lehman *et al.,* 2010). Thus, this ARC cell group co-localises neuropeptides that are both stimulatory (kisspeptin) and inhibitory (dynorphin) to GnRH secretion (Pillon *et al.,* 2003, Dungan *et al.,* 2006;

Kauffman *et al.,* 2007, Lehman *et al.,* 2010) and are thus called KNDy cells (co-localising kisspeptin, neurokinin B and dynorphin; Cheng *et al.,* 2009, Lehman *et al.,* 2010a).

Endogenous Opioid Peptides (EOP)

Endogenous opioid peptides (EOP) have been implicated in mediating the feedback actions of oestradiol on GnRH release in several species, including the rat (Karla 1993), and ewe (Walsh and Clarke 1996). In this situation opioids have been regarded as a 'brake' that must be removed before the GnRH surge can be initiated (Karla 1993). However there is also evidence that the EOP are involved in the inhibitory action of progesterone on GnRH release (Yang *et al.* 1988, Wisnant and Goodman, 1988). Opioid peptides can alter GnRH release via the three main receptor subtypes μ, κ and δ (Malven 1999, Horton *et al.* 1987). Although all three major classes of EOP, the endorphins, enkephalins and dynorphins, have been shown to influence gonadotrophin secretion, the role of each specific one, the hypothalamic site and the mechanism by which they exert their inhibitory action are still largely unresolved. EOP are also involved in stress responses (Foradori *et al.* 2005)

Out of all the opioid compounds found in the brain, the most attention has been given to the reproductive role of dynorphin (Goodman *et al.,* 2004). Historically all three classes of opioids have been implicated in progesterone negative feedback, with most recent being dynorphin (Goodman *et al.* 2004, Dufourny *et al.* 2005). Dynorphin A is a member of a family of opioid peptides derived from the preprodynorphin precursor (PPD). PPD mRNA-expressing cells were seen in the supraoptic nucleus (SON), AHA, bed nucleus of the stria terminalis (BNST), VMN, dorsomedial nucleus of the hypothalamus, and the ARC. All of these regions also contained dynorphin A -positive cell bodies except for the VMN, raising the possibility that PPD is preferentially processed into other peptide products in the VMN (Foradori *et al.* 2005). Consistent with the role as steroid responsive cells, dynorphin cells in the sheep POA, AHA, and ARC have been shown to contain PR (Foradori *et al.* 2002). Dynorphin may also play a role in response to stress as a variety of stressors increase PPD mRNA expression in the PVN of sheep (Matthews *et al.* 1993).

There is evidence that β-endorphin is the most important opioid for oestradiol positive feedback for the generation of the GnRH surge. Its location is thought to be the ARC exclusively (Lehman and Karsch 1993, Taylor *et al.* 2007). Lehman and Karsch (1993) reported that 15±20% of ACTH/b-endorphin cells, located in the medial portion of the ARC, of the ewe possess ERα, while the β-endorphin content in the ARC/ME is lower during the surge than 10–12 h before (Domanski *et al.* 1991). This decreased release of β-endorphin by the hypothalamus during the follicular phase may be an important factor enabling a preovulatory release of GnRH and LH in the sheep (Pillon *et al.* 2003). Furthermore, ovine β-endorphin neurones project from the ARC directly to the POA (Dufourny *et al.* 2005) where the majority of GnRH cells are located (Lehman *et al.* 1986). It should be noted, that in most studies, the method used to evaluate the role of β-endorphin, is the expression of pro-opiomelanocortin (POMC) gene. However, other POMC-derived peptides could be also involved in the control of GnRH or sexual behaviour in the ewe such as a-melanocyte stimulating hormone (a-MSH) known to be capable in female rat to inhibit LH secretion when administrated in the mPOA or in the ME (Gonzalez *et al.* 1997) and to regulate sexual behaviour (Gonzalez *et al.* 1993). Recently Pillon *et al.* 2003 reported that, in the ewe, a decrease of POMC mRNA occurs before the preovulatory surge and could be involved in the positive feedback or in the negative feedback induced by oestradiol occurring before the surge but that the variations of POMC mRNA that were observed could be also related to other events occurring at the time of the LH preovulatory surge such as sexual behaviour, growth hormone (GH) or prolactin preovulatory surges. The previous study, however, is contradicted by Walsh *et al.* (1998) who report that at the peak of the GnRH surge in POMC gene expression is increased in the ARC of intact ewes, respectively. There are several hypotheses for the role of elevated β-endorphin at the peak of the GnRH surge: 1) its role is to terminate the GnRH surge. This would not have any impact on the LH surge as this has been shown to end well in advance of the fall in GnRH (Moenter *et al.* 1990). 2) Its role is to terminate receptive behaviour at times when mating would not lead to conception (Taylor *et al.* 2007). 3) Increased β-endorphin release prior the surge acts at an inhibitory neurotransmitter to suppress the activity of a second inhibitory neural system that impinges on the GnRH neurones. Thus, the increase in β-endorphin would result in disinhibition of the GnRH neurones and result in the GnRH surge. One

possible neurotransmitter that might fill the role of an intermediary inhibitory neurone is GABA (Robinson and Kendrick 1992). 4) β-endorphin has no role in the surge-generating mechanism and controls only pulsatile GnRH secretion (Taylor *et al.* 2007).

Progesterone increase accompanies POMC reduction in cells that express steroid receptors and therefore β-endorphin is also a candidate for mediating progesterone negative feedback (Wisnant *et al.* 1992, Taylor *et al.* 2007). In contrast the more lateral areas of the ARC where there are very few, if any steroid receptive β-endorphin neurones did not show any change in POMC gene expression with increased progesterone concentrations (Taylor *et al.* 2007). The role of these cells remains unknown.

Met-enkephalin is found in the paraventricular nucleus (PVN) and VMN (Taylor *et al.* 2007). Recent information shows that there are sex- and steroid-associated differences in the met-enkephalin neurones in the PVN and suggests that they may have an important role in the control of LH release (Rivalland *et al.* 2005). Taylor *et al.* 2007 report that an increase in progesterone is accompanied by a reduction of PENK (precursor for met-enkephalin) expression in the PVN and are therefore candidates for mediating progesterone negative feedback (Malven, 1999). The link between enkephalin neurones in the PVN and GnRH neurones is less clear. In the case of PENK although there are reports of a decline in mRNA expression in the VMN between luteal and follicular phase intact ewes (Walsh *et al.* 2001), this is not thought to play a role in oestradiol positive feedback or in the control of oestrus behaviour (Taylor *et al.* 2007).

Recently Orphanin FQ (OFQ) a member of the EOP family has been implicated in reproductive endocrinology. OFQ does not exhibit appreciable binding affinity for classical opioid receptors rather it functions as an endogenous ligand for the opioid receptor like (ORL)-1 receptor (Mogil and Pasternak 2001). OFQ cells are present in the POA, AHA, and ARC of the hypothalamus of the rat (Neal *et al.* 1999). In this species this peptide is implicated in feeding and sexual behaviour as Sinchak *et al.* (1997) have shown that OFQ delivered into the VMN facilitates lordosis in female rats in a dose-dependent manner. Interestingly, Foradori *et al.* 2007 report that dual

immunohistochemistry revealed an exceptionally high percentage of GnRH-immunoreactive cells that colocalized OFQ. In addition, intracerebroventricular (icv) infusion of an OFQ agonist inhibited LH pulse frequency in both luteal phase and OVX ewes and suppressed pulse amplitude in OVX ewes. Taken together, these observations raise the intriguing possibility that OFQ may be acting as ultrashort feedback loop to synchronize GnRH pulses (Foradori *et al.* 2007).

Neurokinin B (NKB)

Neurokinin B is a member of the tachykinin neuropeptide family and is abundant in the ARC of many species (Goubillion *et al.,* 2000, Latronico, 2009). Dual-labelling experiments have shown that almost all NKB neurones (97%) express ERα (Goubillon *et al.* 2000) and the analysis of female and male brains has revealed an exceptionally marked female-dominant sex difference in the number of NKB neurones in the ARC (Goubillon *et al.* 2000). Central administration of a neurokinin B receptor agonist stimulates LH secretion in sheep and monkeys (Billings *et al.,* 2010, Ramaswamy *et al.,* 2010), whereas in humans, mutations of either neurokinin B or its receptor (neurokinin 3 receptor; NK3R) are associated with gonadotrophin deficiency and pubertal failure (Topaloglu *et al.,* 2009). Moreover, many Neurokinin B-immunoreactive fibres have been found in close proximity to approximately 40% of the GnRH neurones located in the POA and also intermingled with GnRH fibres in the ME (Goubillon *et al.* 2000). All this anatomical evidence strongly suggests a role for the oestradiol-sensitive NKB cell population in the regulation of female reproductive function. Pillon *et al.* (2003 b) report that 4 h of oestradiol treatment, which is sufficient to induce the preovulatory-like LH surge and oestrous behaviour in progesterone primed OVX ewes, decreases both the level of preprotachykinin B (PPTB) mRNA expression in the NKB neurones of the ovine caudal part of the ARC of OVX ewes, and also the number of cells containing NKB mRNA. Interestingly, the decrease of NKB mRNA expression coincides with the time of the negative-feedback effect of oestradiol on GnRH secretion (Caraty *et al.* 1989) or closely precedes the induction of oestrous behaviour. Recent evidence shows that in the ARC of the ewe, essentially all kisspeptin neurones express dynorphin, and most also express NKB. In contrast, these cells do not produce g-MSH or AGRP (Goodman *et al.* 2007). A single subpopulation of neurones in the ARC contains all three neuropeptides.

Moreover, this subpopulation appears to be distinct from the orexigenic (producing AGRP and NPY) and anorexigenic (producing melanocortins and β-endorphin) neurones in this nucleus. Even though neurokinin B appears to be a stimulating factor of the GnRH/LH surge in the ewe, the mechanism by which this may be achieved is not yet clear as NK3R are not co-localised in GnRH neurones but on KNDy cells (Amstalden *et al.,* 2009). A proposed model for the actions of neurokinin B to control KNDy cell activity is outlined in Lehman *et al.,* (2010) and Maeda *et al.,* (2010), suggesting that neurokinin B/NK3R signaling plays a role in facilitating and synchronizing activation of kisspeptin neurones. This mode of secretion is described for the control of GnRH pulses; however, a similar mode of action could also be employed for the control of the GnRH surge mechanism.

γ- aminobutyric acid (GABA)

GABA is thought to be a major inhibitory neurotransmitter with a widespread distribution throughout the mammalian central nervous system. GABA contains steroid receptors (oestradiol and progesterone) and is extremely sensitive to their change in concentration. Moreover, close contacts between GABAergic terminals and preoptic GnRH cell bodies were identified in sheep (Herbison *et al.* 1993). Numerous studies have shown that administration of GABA, or GABA agonists and antagonists, alters LH secretion in ewes (Sliwowska *et al.* 2006), while in the mPOA, activity of the GABAergic system declines significantly just before the GnRH surge onset (Robinson *et al.* 1991). This is done by the uncoupling of the normal stimulatory influence of noradrenaline (NA) on GABA neurones at the time of the surge, which thereby allows an increase of the stimulatory influence of NA on GnRH neurones (Robinson *et al.* 1991, Herbison 1998). Therefore it is suggested that the mPOA in ewes is an important regulatory site of GABA on GnRH release. GABA, affects GnRH secretion by two different classes of membrane receptors: $GABA_A$ receptors and $GABA_B$ receptors (Bormann, 2000). Numerous experiments indicate that GABA may mediate both inhibitory as well as stimulatory effects on GnRH release through a $GABA_A$ receptor mechanism. The most reasonable explanation for this contradictory role may be that $GABA_A$ receptors are located on numerous stimulatory and inhibitory interneurones that in turn influence GnRH neurones. Tomaszewska-Zaremba et al. (2003) suggest that suppression of GnRH during

$GABA_A$ receptor activation might be viewed as the net effect of inhibitory and stimulatory actions, i.e. of a direct inhibitory effect on GnRH neurones, indirect suppressive influence on GnRH release by activation of β-endorphinergic systems, and indirect activation of GnRH cells by increasing NA release. GABA's role is not however limited to the mPOA as quantitative immunocytochemical analysis revealed that the majority of axosomatic terminals in the ARC are GABAergic, and this population of synapses is affected by oestradiol (Kurunszi *et al.* 2008). However, the mode of action of GABA in the control of GnRH release is still far from being understood.

Neuropeptide Y

The implication of neuropeptide Y (NPY) in the hypothalamic regulation of LH and GnRH surges is supported in several mammalian species, notably in the rat (Kalra *et al.* 1995). In rats and sheep NPY neurones are found in the ARC of the hypothalamus (Antonopoulos *et al.* 1989) in addition to other areas in the brain and projections from NPY neurones in the ARC have been reported to contact GnRH neurones in the POA (Tillet *et al.* 1989). NPY immunoreactive fibres are also seen in close association with GnRH terminals in the external zone of the ME of the rat (Estrada *et al.* 2003). Thus it is possible that NPY could exert control over GnRH secretion by action on GnRH cell bodies or terminal fields. Up to 10% of NPY neurones in the ARC contain ER (Skinner and Herbison 1997) allowing for feedback effects of oestradiol on GnRH secretion to be mediated via a subset of this neuronal cell type. In the ewe, the results seem quite conflicting. Malven et al. (1995) showed in ovariectomized ewes that icv administration of anti-NPY antibodies around oestradiol treatment advanced the onset of the LH surge without changes in its magnitude, whereas Porter *et al.* (1993) claimed that in intact cyclic ewes, icv injection of anti-NPY antibodies prior to and during the expected time of the preovulatory surge delayed or abolished the LH surge, with an inhibition of oestrous behaviour. Pillon *et al.* (2003) demonstrated that a 4-h (proven to be enough to stimulate an LH surge and the onset of sexual behaviour) oestradiol treatment induces no change for preproNPY mRNA, in the infundibular nucleus (IN) of ovariectomized progesterone-primed ewes. Therefore as NPY content of the MBH increases just before and remains elevated during the preovulatory surge in ewe (Malven *et al.* 1995) this modification in mRNA expression should be able to be detected later than the time of

oestradiol action (Pillon *et al.* 2003). Estrada *et al.* (2003) measured NPY mRNA expression in the ARC across the oestrous cycle and found that levels were reduced in the caudal ARC during the preovulatory surge period compared to the luteal phase levels, which is in contrast with the rat. Furthermore, to test the hypothesis that NPY is a predominant negative regulator of GnRH secretion they infused NPY in the third ventricle and this caused a delay in the LH surge in OVX ewes. They conclude that NPY is a predominant negative regulator of GnRH secretion. Studies in rats suggest that NPY has a stimulatory role in the generation of the preovulatory GnRH surge via the Y1 receptor although in sheep antagonism of the specific receptor did not block the surge (Estrada *et al.* 2003) and therefore influence may be exerted via a different receptor, e.g. Y2 or Y3 or Y4 or Y5 in this species. In the ARC of the ewe, there is a marked alteration in the expression of NPY, with alteration in bodyweight. Reduction of body weight by food restriction in OVX ewes increased NPY mRNA expression (Adam *et al.* 1997, Henry *et al.* 2000). This peptide promotes feeding in the sheep, suggesting involvement in homeostatic control. NPY also stimulates the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) and may therefore be fundamentally involved in `stress' responses (Clarke *et al.* 2001).

Somatostatin

Within the mediobasal hypothalamus (MBH) of the ewe, numerous neurones synthesizing somatostatin are present in the infundibular nucleus (IN) and the ventrolateral division of the (VMN; Bruneau and Tillet 1998). However, the physiological role of the somatostatin-containing neurones remains undefined. Colocalization studies have shown that about 70% of the neurones expressing oestrogen receptors in the VMN synthesize somatostatin, and 13% of the somatostatinergic neurones located in the IN express ERα (Scanlan *et al.* 2003). Therefore, the cells synthesizing somatostatin located in the VMN and/or the IN might play a role in the induction of the preovulatory GnRH surge or sexual behaviour. Pillon *et al*. (2004) demonstrated that icv administration of somatostatin, which simulates the increase in PPS mRNA expression observed by in situ hybridization after the oestradiol treatment, totally inhibited the pulsatility of LH secretion. The increase of PPS mRNA expression coincides with the time of the negative feedback that oestradiol exerts in the central nervous system during the preovulatory

period (Caraty *et al.* 1989) and closely precedes the induction of oestrous behaviour. Moreover, as preovulatory oestradiol replacement by somatostatin icv infusion abolished LH pulsatility and dramatically decreased the mean basal level of LH secretion, we hypothesized that somatostatin may be involved in the negative feedback of oestradiol.

Galanin

Galanin is mainly observed in the mPOA and the ARC. The highest density of immunoreactive fibres was found in the external layer of the ME. Numerous galanin-immunoreactive fibres were also observed in the POA, the MBH, the periphery of the SON and PVN. With colchicine treatment, the number of labelled neurones increased, and additional galanin-immunoreactive perikarya were observed in the BNST, the lateral septum, the SON, the PVN and the PeVN and the PVN of the thalamus. (Chaillou *et al.* 1999). Early studies performed both in the rat have shown that galanin can stimulate and modulate GnRH secretion from the hypothalamus and stimulate LH release from the anterior pituitary gland (Grafsteindunn *et al.* 1994). Furthermore, rodent studies have shown that oestrogen can stimulate galanin gene expression (Gabriel *et al.* 1993) and that galanin might act presynaptically to shape and modulate GnRH pulses (Grafsteindunn *et al.* 1994). It has subsequently been shown that essentially all GnRH expressing neurones in the ovine hypothalamus coexpress galanin, although not all galanin-expressing cells coexpress GnRH (Dufourny *et al.* 2003). Recent research on the expression of galanin in sheep has shown that over 50% of the galanin-expressing neurones of the POA of the ovine hypothalamus also express ERα (Tourlet *et al.* 2005).

Noradrenaline

Almost all noradrenergic cells are found in the brain stem and specifically in the regions A1 (ventrolateral medulla; VLM) and A2 (nucleus of the tractus solitarius; NTS). Noradrenergic cells located in A1 and A2 cell groups in the brainstem are thought to be of importance, for the generation of the surge in GnRH/LH secretion. NA-producing cells of the brain stem are involved in regulating GnRH cells and project to the POA and BNST (Barraclough and Wise 1982). In rats and sheep, the NA cells that project to the POA, express ERα and are activated by oestradiol (Rawson *et al*. 2001). In sheep, the A1 cells

provide the most significant NA input to the POA and activation of the A1 neurones (expression of c-Fos protein) is observed in OVX ewes within 1 h of oestradiol injection (Rawson *et al.* 2001). Although only 20% of GnRH cells appear to receive input from NA systems in the ewe (Pompolo *et al.* 2003) NA could also regulate these cells by relay via other forebrain regions. NA role is to stimulate GnRH secretion, but on the other hand NA axons also synapse directly or indirectly with GABA perikarya resulting in suppression of GnRH secretion. Therefore oestradiol, via brain stem NA, can both stimulate and suppress GnRH secretion. Together with opioids they control pulse amplitude. NA concentrations are elevated before the LH surge in the POA and ME while ablation of the NA pathway can block the LH surge. After this ablation the LH surge mechanism recovers fully giving evidence that there are compensatory mechanisms and NA has a 'permissive' role towards the GnRH release (i.e., it should be considered as a modulator that enables interactions between other neurones rather than a stimulator or inhibitor (Dobson *et al.* 2003, Smith and Jennes 2001). It has been recorded that the infusion of NA in the 3V inhibits the release of LH pulses in OVX rats but this inhibition is reversed when they were treated with oestradiol. It would be of great interest to determine which and if in fact there is a threshold of oestradiol concentration, which causes this 'switch'.

Glutamate (Excitatory Amino Acids; EEA)

Glutamate is the major endogenous EAA in the CNS that mediates excitatory synaptic neurotransmission through EAA receptors (Brann and Mahesh 1997). Glutamatergic inputs to GnRH cells have been demonstrated histologically, while glutamate fibers have been found in close apposition to GnRH cells in the ewe POA (Pompolo *et al.* 2003). Studies have revealed that within the hypothalamus of the rat and monkey, dense glutamate immunostaining is found in the magnocellular and parvocellular PVN, VMN, SON, LHA, suprachiasmatic nucleus (SCN), ARC, infundibular stalk, and ME (Brann and Mahesh 1997). Pompolo et al. (2003) used an antibody to vesicular glutamate transporter 2 (VGluT2) to label glutamatergic neurones (as there is a problem in identifying the location of the glutaminergic cell bodies as all cells use glutamate for biosynthetic purposes) in the areas of the ewe brain that control GnRH secretion. VGluT2- immunoreactive cells were observed in the ARC, VMN, POA, BNST, and A1 and A2 cell groups in the brainstem. As a whole, these immunolocalisation studies

demonstrated that glutamate is extensively localized in presynaptic terminals throughout the vast majority of the nuclei of the hypothalamus, and hence strategically positioned to control many different functions of the hypothalamus. Microdialysis studies by Ping *et al.* (1994) in ovariectomized adult rats have shown that immediately before peak serum LH levels there was an enhanced release rate of glutamate in the POA and therefore support that EEA are necessary for the activation of GnRH neurones. Significant numbers of glutamatergic neurones were found to express ERα, including those of the ARC, VMN, which are known to be activated by oestradiol and project to POA where most of GnRH neurones are located. Their input may be direct or indirect. Approximately 40% of glutamatergic cells in the BNST/POA express ERα, allowing for indirect oestrogenic regulation of GnRH cells (Pompolo *et al.* 2003). 50% of GnRH neurones that express c-Fos during the LH surge have glutamate receptors (Dobson *et al*. 2003). Other factors that have been demonstrated to modulate EAA release from the hypothalamus include the neurotransmitter GABA, which has been shown to stimulate glutamate release from rat POA neurones via a $GABA_A$ receptor mediated mechanism (Fleischmann *et al.* 1995).

Sexual Behaviour

The increase in the concentration of oestradiol in the mid-follicular phase is also responsible for changes in sexual behaviour (Fabre-Nys and Gelez 2007, Karsch *et al.* 1980). In order for sexual behaviour to occur, increasing concentrations of oestradiol must follow a period of exposure to progesterone, but the latter must no longer be present in the bloodstream (Fabre-Nys and Martin 1991). In order to determine steroid requirements for the occurrence of sexual behaviour, numerous studies have used an ovariectomised – steroid replacement ewe model. In this model, where sexual behaviour does not occur, administration of oestradiol alone is enough to induce both the LH surge and receptive behaviours (Fabre-Nys and Martin 1991). However, full expression of oestrus is exhibited only with the previous administration of progesterone for a minimum of 3 days. Progesterone priming also increases the proportion of females exhibiting oestrus, reduces the time to onset of sexual behaviour, and increases its duration and intensity (Fabre-Nys and Martin 1991).

Depending on age, breed, and the time of year the interval between two oestrous periods in domestic ruminants is 17 days and the duration of oestrus expression 6 to 36 h (Fabre-Nys *et al.* 1993). In all cases, these changes in behaviour occur shortly before the release of the preovulatory LH surge which triggers ovulation.

Beach (1976) proposed a useful classification of female sexual behaviour in to 3 components: attractivity, proceptivity and receptivity. Attractivity refers to the female's value as a sexual stimulus to the male. Olfaction is certainly important here, as sexually experienced rams are able to discriminate between the smell of an oestrous female and a non-oestrous one. Proceptivity consists of appetitive activities shown by females such as ram seeking activity which is displayed by 75% of ewes. In addition females can display specific behavioural motor patterns which will increase the males' interest. These patterns include movement of the head toward the male, and tail fanning, but the main display that they show is just to stand still near the male (Fabre-Nys *et al.* 2007). Receptive behaviour consists of an “active immobilization” during which the female will resist if you try to push her. In the ram, intromission only lasts a few seconds and is followed by ejaculation (Banks 1963). There is no need for a series of intromission although the reproductive success of the ewe is enhanced if mating occurs more than once (Synnott *et al.* 1981).

The preovulatory GnRH secretion, measured in portal blood and cerebrospinal fluid, starts at the time of the LH surge, approximately 4 h after the onset of oestrous behaviour, and lasts as long as receptivity (36–48 h), which is much longer than the LH surge. Caraty *et al*. (2002) administered exogenous GnRH or GnRH antagonist and demonstrated that the sustained secretion plays a physiological role in extending the duration of oestrous behaviour by controlling receptivity. The same authors also suggest that during the oestrous cycle of the ewe at least two mechanisms are necessary for the expression of sexual behaviour around the time of ovulation: one involving oestradiol and one involving oestradiol-induced GnRH secretion.

It has been suggested that steroid mediated oxytocin release, has an inhibitory role in controlling female sexual receptivity. In most mammals, a peripheral and central

increase of oxytocin release is observed after repeated intromissions or manual vaginocervical stimulation and is followed by a shortened oestrous expression. In addition, infusion of oxytocin into the MBH, of receptive female ewes significantly decreases sexual receptivity (Kendrick *et al*. 1993). Administration of this neuropeptide induces an increase of NA in the MBH and it is therefore considered that they act synergistically to exert an inhibitory effect on oestrous behaviour (Fabre-Nys and Gelez 2007).

Steroid hormones are believed to stimulate the onset of female sexual responses by modulating neurotransmitter systems. However, the effect of neurotransmitters on the control of female oestrus behaviour has not yet been clearly defined. DA and NA have received most attention and are considered to be the main regulators (Fabre-Nys and Gelez 2007, Fabre-Nys *et al.*1994). Microdialysis measurements have shown that the major neurochemical changes, associated with steroids, in the MBH during oestrus, involve these two neurotransmitters (Fabre-Nys *et al.*1994). NA increases transiently during periods of sexual receptivity and just following sexual interactions with a male (Fabre-Nys *et al.* 1997). Furthermore, antagonism of NA in the MBH reduces proceptive behaviour, and administration of NA enhances proceptive behaviour in sheep (Fabre-Nys *et al.,* 2003). DA has a biphasic role and facilitates the expression of oestrus behaviour when high before oestradiol but is inhibitory after oestradiol (Fabre-Nys *et al.* 2003). The fact that these patterns of changes in DA levels are consistent only when ewes are going to become fully receptive suggests that they are related to the onset of sexual behaviour (Fabre-Nys *et al.* 1994). Changes in NA are thought to be modulated by DA transmission in the MBH as quinpirole (a DA receptor agonist) administered before oestradiol increases the NA release, while quinpirole administered after oestradiol reduces it (Fabre-Nys *et al.* 2003). A few other neurochemical changes have been observed in association with oestrous behaviour in sheep such as a decrease in serotonin and GABA. But the role of these substances has not been studied further in domestic ruminants.

Stress

Stress can have deleterious effects on metabolism, growth, tissue repair and reproductive physiology. There are many different types of stress: physical (e.g. transport), immunological (e.g. administration of endotoxin) or psychological (e.g. isolation; Dobson *et al.* 2003) which all activate the hypothalamic – pituitary – adrenal axis (H-P-A axis). This activation leads to the reduction of GnRH and therefore LH pulsatility (frequency and amplitude) by interfering with both the hypothalamus and the pituitary gland resulting in a delayed or even absent LH surge (Smith *et al.* 2003). This can be accomplished through direct influence on GnRH secretion mechanisms or/and indirectly by the induced decrease in oestradiol production caused by impaired follicular growth. In monkeys, administration of oestradiol can reverse the effects indicating that there is a dose effect and that the HPA response is mediated by the steroid hormone milieu. This remains to be proven in other species as well to eliminate the possibility of a species-specific response (Dobson *et al.* 2003). It is therefore evident that stressors disrupt the correct function of all parts of the H-P-O axis (Dobson *et al.* 2000).

Depending on their intensity, stressors act on the activation and/or transmission phases of the GnRH surge, by stimulating the PVN. Substantial pathways from the PVN to the central VMN and the lateral ARC have been identified (Qi *et al.* 2008) and this route may directly or indirectly disrupt GnRH cell recruitment.

Adrenal stimulation is the result of increased ACTH release from the pituitary gland. This release is modulated by the activation of the PVN to produce two factors: corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) that reach the ME (there, they do not alter GnRH secretion for it has been found that their terminals are not closely associated with GnRH neurones and are secreted in the hypophyseal portal system (Dobson *et al.* 2003, Smith and Dobson 2002, Tellam *et al.* 2000, Rivier and Rivest, 1991). CRH is thought to predominate during 'weak' stressors while additional AVP is secreted during more intense stress. Even though AVP concentrations are higher in the ewe compared to CRH, AVP antagonists do not reverse the effects, while CRH antagonists do

(Rivalland *et al.* 2005) indicating that both of them participate in suppression and CRH's role may be more important. It is possible that CRH exerts its effects on GnRH pulse frequency via GABA – NA neurones, whereas AVP responses involve opiodergic cells (i.e. pulse amplitude) as has been proven in rats. The activation of the PVN is induced by prior activation of the brain stem to produce NA and NPY and the ARC to produce NPY. Oestradiol enhances stress induced NA suppression (when applied to the brain stem and PVN, not mPOA and ARC). The PVN also receives dopaminergic projections from the area postrema and GABA projections from the mPOA

The activated CRH and AVP neurones have many tasks: to stimulate ACTH release, to decrease pulse frequency via direct synapse with GnRH neurones (Jasoni *et al.* 2005) and to activate GABA and opioid neurones which they in turn influence GnRH neurones (Tellam *et al.* 2000). CRH neurones from the PVN are thought to project to GABA cells in the mPOA. This is contradicted in recent studies by Rivalland *et al* (2005) who used retrograde tracing and c-Fos labeling to prove that there are no direct synapses from the PVN to the mPOA. Recently Jasoni *et al.* (2005) have identified approximately 30% of GnRH neurones in the mouse, spread throughout the GnRH neurone continuum, expressed CRH receptor immunoreactivity and are therefore direct targets of stress responses. This remains to be proven in other species such as the sheep.

Another stress pathway involves amygdala (CeA, plays a role in emotional behaviour and motivation), which contains CRH neurones that project to the mPOA via the BNST, and increase GABA during stress (Rivalland *et al.* 2005). Stressors that require limbic interpretation could activate this pathway. In the rat destruction of the PVN did not reverse the effects of stress providing strong evidence of alternative stress pathways.

Under certain conditions CRH can up-regulate (Battaglia *et al.* 1998) LH release or have no effect on it but only during periods of oestradiol negative feedback. Maybe this provides proof that not all populations of CRH neurones in the brain have the same role. This finding could be the result of neurotransmitter action unconnected to the stress axis or steroid feedback mechanisms may be important.

ACTH released from the pituitary gland has an effect on the LH surge and this is thought to be mediated by progesterone secreted by the adrenal glands. Progesterone's deleterious effects are in turn mediated by opioids from the ARC, which project to the mPOA and ME. It should be noted that administration of a progesterone antagonist did not reverse the disruption of LH (Smith *et al.* 2003, Smith and Dobson 2002).

There is contradiction on the role of the corticosteroids released from the adrenal gland medulla under the influence of ACTH. Several studies show that they are not likely to have a major influence, since effects have been also observed in adrenalectomized animals as well as the fact that after the administration of an antagonist effects are still observed (Rivalland *et al.* 2005, Smith and Dobson 2002), while their chemical nature does not allow them to pass the blood – brain barrier. Furthermore, corticosteroids are elevated for only a limited period of time due to negative feedback mechanisms on ACTH release resulting in their decrease even though the stressor is still being perceived by the animal (Smith and Dobson 2002). On the other hand, administration of a high dose of cortisol did cause LH secretion abnormalities (Dobson *et al.* 2003) while in some articles their role is thought to inhibit GnRH production at the level of the ME and to alter responsiveness at pituitary gland level (Tellam *et al.* 2000; mainly by binding with glucocorticoid receptor (GR) type II). These lead us to believe that corticosteroids are in fact mediators of stress effects but whether or not they are necessary still remains to be proven. Macfarlane *et al* (2000) contradict the former statement and support that corticosteroids are in fact necessary at least in the OVX ewe, and imply that stress-like concentrations were not achieved in previous studies. Furthermore they state that when the appropriate level of concentration is achieved there is also a reduction in LH pulse frequency (in intact animals) indicating that there is influence at hypothalamic level. They propose that the type II GR found in hypothalamic neurones are responsible for this decrease, mediated by the steroidal milieu.

Interestingly, a recent study on female macaques reports that 'stress sensitive' and 'stress resilient' individuals exist and that these individuals in the absence of stress have 1) fewer serotonin neurones, 2) lower expression of 5HT2A and 2C genes (serotonin

receptors) in the PVN and IN 3) higher gene expression of GAD67 and CRH and 4) reduced GnRH transport to the pituitary (Bethea *et al.* 2008).

The effect of stress on the exhibition of oestrous behaviour has received little attention. Recently Pierce *et al.* (2008) used a layered paradigm of psychosocial stressors and report a reduction in the intensity of attractivity and proceptivity expression. Interestingly receptivity was unaffected by stress. This provides evidence that the expression of various behaviours are controlled by different neuronal mechanisms.

Endotoxin

Specifically, immune/inflammatory challenges, such as bacterial endotoxin (i.e. lipopolysaccharides; LPS), disrupt the ovulatory cycle in sheep, by interfering with the GnRH, and therefore the LH, surge in OVX and intact ewes (Breen *et al*. 2004, Battaglia *et al.* 2000). After endotoxin administration a rise in CRH and AVP in the portal blood and cortisol and progesterone in the peripheral blood was observed while the GnRH and LH pulsatility was suppressed (i.e. the HPA axis was activated; Karsch and Battaglia 2002, Battaglia *et al.* 1998, Battaglia *et al.* 1997). The much higher concentration of AVP compared to CRH agrees with the level of severity of the stressor. Although the exact mechanism by which this is achieved is not clear, endotoxin has the ability to disrupt the oestradiol activation phase (phase I) of the GnRH surge and not the transmission or release phases (phase II and III; Battaglia *et al*. 1999). It is suggested that this is done by action upon neuroendocrine centres that respond to the positive feedback signal of oestradiol, which leads to the GnRH surge (Karsch and Battaglia 2002, Battaglia *et al*. 1999). Interestingly, Battaglia *et al* 1998*,* state that administration of endotoxin affects LH amplitude and frequency while at hypothalamic level there is only a decrease in GnRH pulse amplitude in ovx ewes. This indicates that there is an uncoupling of GnRH and LH pulsatile secretion. In the same study it is also noted that GnRH overcame suppression quicker than LH suggesting that pituitary response was compromised. In another study (Battaglia *et al.* 2000) administration of endotoxin disrupted oestradiol rise, blocking the LH surge but did not inhibit LH pulses in half of the animals tested indicating that endotoxin may also act at ovarian level impairing follicular development or/and inhibiting oestradiol secretion in response to gonadotrophic stimulation. Smith *et*

al. 2003 suggest that low doses of endotoxin act on the pituitary gland while high doses act on both the hypothalamus and the pituitary gland to suppress both pulsatile and surge secretion. Recently a study in rats investigated the effect of LPS administration on kisspeptin gene expression. In this study LPS injection decreased hypothalamic kisspeptin mRNA expression as well as plasma LH levels while indomethacin completely blocked the suppressive effects of LPS on LH secretion and kisspeptin mRNA level. And therefore conclude that kisspeptin systems are sensitive to immune/inflammatory challenge conditions and transmit these signals into the central reproductive system (Iwasa *et al.* 2008).

Insulin

Reproductive maturation and function is sensitive to the state of energy reserves of the organism (Fernandez-Fernandez *et al.* 2006, Tena-Sempere, 2007). The basis for alterations remains partially unknown, but it is likely to involve multiple interactions between the neuroendocrine networks governing energy balance and GnRH function that take place at different levels of the H-P-A axis (Fernandez-Fernandez *et al.* 2006). Although physiological amounts of insulin are necessary for neurone activity, the sudden administration of a bolus insulin injection activates the H-P-A axis due to the induction of hypoglycaemia (Dobson *et al.* 2003, Smith *et al*. 2003, Ohkura *et al.* 2000, Caraty *et al.* 1990). Hypoglycaemia blocks or delays the LH surge when applied to any of the three phases of the surge mechanism (i.e. activation, transmission and surge). The delay in LH surge onset that is observed after blood glucose decreases below the 3 mmol l^{-1} threshold (Dobson and Smith 2000), is thought to be mediated via opiodergic action on the hypothalamus (Smith *et al*. 2003, Clarke *et al*. 1990). The activation and the release of CRF have also been implicated (Dobson and Smith 2000, Adam and Findlay 1998). The glucose-sensing sites that mediate the LH suppression are considered to be central and not peripheral (liver; Ohkura *et al.* 2000) but the exact location and neurone identity have yet to be determined in sheep. Intriguingly, in humans there is recent evidence that the MBH itself is a control centre of satiety and contains glucose-excited and glucose-inhibited neurones that, at least in part, overlap with the neuronal populations that express β-endorphin, AGRP, a-MSH and NPY (Mountjoy and Rutter 2007, Penicaud *et al.* 2006). This means that their electrical activity is altered in response to increasing

and decreasing glucose concentrations as well as the hormone insulin itself. It is also mentioned that these glucose sensitive neurones may be involved in raising blood glucose levels (i.e. they 'sense' low levels of glucose). Apart from the involvement of NPY studies have shown the galanin neurones (Cunningham, 2004), steroidogenic factor-1-expressing neurones in the VMN of rats (Qi *et al.* 2008) and POMC neurones (which also contain insulin receptors; Qi *et al.* 2008, Schioth and Watanobe 2002) have dual effects to regulate metabolism and reproduction. Thus, the multifunctional properties of NPY, POMC, steroidogenic factor-1 and galanin neurones indicate the close relationships that exist between the regulation of food intake and reproduction. Once again there is a hypothesis that kisspeptin may participate in transmitting metabolic information on to the centers (namely, GnRH neurones) (Tena-Sempere 2007). The initial studies linking energy status and the kisspeptin system were focused in the analysis of changes in kisspeptin 1 gene expression in situations of metabolic stress. Thus, in pubertal male and female rats, protocols of acute fasting (up to 72-h) were shown to induce a significant decrease in the relative expression levels of kisspeptin mRNA at the hypothalamus that was associated to the lowering of circulating LH (Castelano *et al.* 2005). In the same study it is suggested that NPY might participate in the metabolic regulation of kisspeptin in the hypothalamus.

Aims of thesis

Successful reproduction requires follicular maturation and oestradiol biosynthesis, induction of the LH surge, ovulation, and expression of sexual behaviour to be coordinated within a tight time frame. The ARC, VMN and mPOA have been identified critical sites for oestradiol and progesterone action on female sexual behaviour (proceptivity and receptivity) as well as the GnRH surge. Therefore, within this structure, there are some cell populations that are able to read the oestradiol signal from the growing follicles and to activate the oestrus behaviour–generating and surge-generating system. The objective of the present thesis is to contribute to the better understanding of the two mechanisms as well as elucidate potential pathways of their disruption after acute stress.

Specifically the aims of the present thesis are:

1. Acute insulin administration causes a disparity between the onset of oestrus behaviour and the LH surge in ovary-intact ewes. In the present thesis we examined a large number of animals to determine the effect of insulin and LPS on the timing of onset of sexual behaviours and the LH surge, especially with respect to each other's onsets, and investigate which hormonal insult is the cause of the disruption.
2. There is a difference between rodents and sheep as to which kisspeptin population is important for mediating oestradiol positive feedback. In rodents the periventricular population has been shown to be necessary for the initiation of the GnRH surge whereas in sheep results are conflicting. We aimed to investigate the activational pattern of kisspeptin cells during the follicular phase of intact ewes and determine the population(s) activated during the surge.
3. The hypothalamic neuropeptide kisspeptin is essential for GnRH/LH surge release and, therefore, ovulation. We aimed to test the hypothesis that inhibition of kisspeptin activation is a major contributing factor to LH surge disruption during stress. If kisspeptin cells co-localise CRFR type 2 this may be a potential inhibitory pathway.
4. We aimed to map the activation pattern of oestradiol receptor α (ERα) containing cells in the arcuate nucleus (ARC), ventromedial nucleus (VMN) and medial preoptic area (mPOA) during the follicular phase of intact ewes as well as determining whether stress-induced disruption of the LH surge involves a reduction of activated ERα-cells.
5. KNDy cells are considered major regulators of GnRH secretion. We mapped the activation pattern of kisspeptin, neurokinin B and dynorphin (KNDy) cells in the middle and caudal ARC during the follicular phase of intact ewes. Furthermore we investigated whether KNDy cells are targets for stressors.
6. We aimed to determine the activation pattern of dopamine, β-endorphin, neuropeptide Y and somatostatin cells in the mediobasal hypothalamus (MBH) during the follicular phase of intact ewes, as well as investigating whether these neuropeptides participate in the stress response to insulin or LPS.

Chapter 2

Chapter 2

Oestrus behaviour, luteinising hormone and oestradiol profiles of intact ewes treated with insulin or endotoxin

Keywords: Sexual behaviour, ewe, LH surge, oestradiol, stress, cortisol.

Abstract

Acute insulin administration causes a disparity between the onset of oestrus behaviour and the LH surge in ovary-intact ewes. To examine the considerable variation in responses, in the present study we used a large number of animals to confirm findings with insulin, and examine whether endotoxin has the same effect. During the breeding season, follicular phases of intact ewes were synchronised with progesterone vaginal pessaries and received saline vehicle (n=22; controls), insulin (4 iu/kg; n=21 ewes) or endotoxin (LPS; 100ng/kg; n=10) at 28h after progesterone withdrawal (time zero). In controls, the LH surge onset occurred at 36.5 ± 5.7 h and first display of oestrus at 38.2 ± 1.8 h, but there was a delay of 17.6 h (P<0.001) and 7.2 h (P<0.05), respectively, in half the insulin-treated animals ('insulin-delayed') but not in the other half; and a delay of 22.5 h (P<0.001) and 20.7 h (P<0.001), respectively, in all LPS-treated animals. Plasma oestradiol concentrations decreased after both stressors, and remained low for a period of time equivalent to the LH surge delay (P<0.001; Rs-q= 78%). Cortisol increased for 12 h after treatment in both insulin subgroups and the LPS group (P<0.05); whereas progesterone increased in the insulin-delayed and LPS groups from 4.0±0.5 ng/ml and 5.3 ± 1.0 ng/ml to a maximum of 5.7 ± 0.3 ng/ml and 8.8 ± 1.6 ng/ml, respectively (P<0.05 for both comparisons). Plasma triglycerides were measured to assess insulin resistance, but concentrations were similar before and after treatment (0.25 ± 0.01 mmol/l versus 0.21 ± 0.01 and 0.25 ± 0.01 mmol/l versus 0.26 ± 0.01 mmol/l in the insulin-non delayed and insulin delayed subgroups, respectively). Therefore, we hypothesise that a) when an acute stressor is applied during the late follicular phase, the duration of the LH surge delay is related to the duration of oestradiol signal disruption b) cortisol is not the key disruptor of the LH surge after insulin, c) insulin (but not LPS) can separate the onsets of LH surge and oestrus by approximately 10 h, providing a model to

identify the specific neuronal systems that control behaviour distinct from those initiating the GnRH surge.

Introduction

For the successful production of offspring, it is essential for sperm to fertilise the ovum at an appropriate time. This is why sexual behaviour occurs shortly before the preovulatory luteinising hormone (LH) surge which triggers ovulation (Fabre-Nys and Martin, 1991, Fabre-Nys and Gelez, 2007). There is considerable evidence that various types of stressors disrupt the follicular phase of the ovarian cycle and block or delay the LH surge (Karsch *et al.,* 2002, Smith *et al.,* 2003, Dobson *et al.,* 2003) by activating the hypothalamic-pituitary-adrenal (H-P-A) axis. However, few studies have investigated the impact of acute stressors on sexual behaviour in sheep, and those have focused mainly on the incidence and timing of mounting (oestrus) and not other components of sexual behaviour which affect the chances of mating (Fletcher and Lindsay, 1968, Tilbrook and Pearce, 1987). For example, thermal stress reduces the frequency of estrus (Sejian *et al.,* 2010) and delays onset (Doney *et al.,* 1973), as does endotoxin (Battaglia *et al.,* 2000). Recently Pierce *et al.* (2008) used a layered paradigm of psychosocial stressors and reported a reduction in the intensity but not timing of attractivity (ability of ewes to attract rams) and proceptivity (the motivation of ewes to seek rams and initiate mating). Interestingly receptivity (i.e. oestrus) as well as the timing of onset of all the above were unaffected by this type of stress. Regarding LH, a plethora of stressors are known to inhibit pulsatile and surge secretion. For example, the LH surge is delayed or even blocked by the combination of exposure to exercise, food restriction and transfer to a novel environment (Bethea *et al.,* 2008), truck transport (Dobson *et al.,* 1999, Phogat *et al.,* 1999), endotoxin administration (Battaglia *et al.,* 1999, Karsch *et al.,* 2002) or insulin-induced hypoglycaemia (Medina *et al*., 1998, Saifullizam *et al*., 2010).

From a mechanistic point of view, sexual behaviour and the LH surge are controlled by the hypothalamic-pituitary-gonadal (H-P-G) axis. In the ewe, as in many other species, increased oestradiol secretion from the ovarian dominant follicle(s) exerts a positive feedback action in the central nervous system to generate the LH surge and ovulation (Moenter *et al.,* 1990, Goodman 1996). The decrease in progesterone concentrations

after luteolysis and the increase of oestradiol is also responsible for changes in sexual behaviour (Karsch *et al.,* 1980, Blache *et al.,* 1991, Fabre-Nys & Martin 1991, Fabre-Nys & Gelez 2007). Stressors can disrupt oestrus and the LH surge by acting on any - or all - of the organs that constitute the H-P-G axis (Smith and Dobson, 2002, Smith *et al.,* 2003). The immunological stressor endotoxin (i.e. lipopolysaccharide; LPS) or the metabolic stressor insulin-induced hypoglycaemia can interrupt the GnRH/LH surge by acting on all three parts of the H-P-G axis: inhibiting pulsatile GnRH/LH secretion at hypothalamic (Battaglia *et al.,* 2000; Suzuki *et al.,* 2001) and pituitary (Williams *et al.,* 2001) level, thereby blocking the preovulatory oestradiol increase; disrupting the process by which oestradiol generates the preovulatory GnRH/LH surge by acting upon neuroendocrine centres that respond to the positive feedback signal (Battaglia *et al.,* 1999, Karsch *et al.,* 2002; Ghuman *et al*., 2011); and suppressing steroidogenesis at the ovarian level (Shakil *et al.,* 1994, Downing *et al.,* 1999, Battaglia *et al.,* 2000).

It has been shown recently that a sudden activation of the H-P-A axis in the late follicular phase, by insulin-induced hypoglycaemia, lowered oestradiol concentrations and delayed the LH surge; whereas, the onsets and frequencies of a range of pre-copulatory behaviours were not affected (Saifullizam *et al.,* 2010). However, in that study, concern was expressed about the great variation in the timing of the LH surge onset after insulin administration. The aim of the present study was to extend the above observations by comparing the effects of two different types of stressors, LPS and insulin, on a larger number of free-running intact ewes. The stressors were applied at 28 h (and a second injection at 30 h, for insulin) in the mid-late follicular phase. We were interested in the role of steroid responses in disrupting the LH surge, pre-copulatory behaviours as well as oestrus, especially concerning respective timings of the onsets, in an intact ewe model.

Materials and methods

Animals, study design and blood sampling procedure

The study was performed in the mid-breeding season (October/November) on 60 mature intact Lleyn crossbred ewes, weighing between 50 and 80 kg. Because of the large number of animals involved, the study was carried out in two identical replicate sessions with new ewes in the second batch. All ewes were penned indoors (space 15 x 7

metres) with 3 teaser rams. All animals were fed daily a constant diet of ad libitum hay and had free access to water. Frequent handling, for at least a week prior to commencement acclimatizing the animals to human manipulation and the blood sampling process ensured that minimal stress responses were incurred. All procedures were conducted in accordance with requirements of the UK Animal (Scientific Procedures) Act, 1986, and approved by the University of Liverpool Animal Welfare Committee.

Ovarian follicular phases were synchronised with the administration of two intravaginal progesterone-releasing pessaries (Controlled Internal Drug Release [CIDR-G]; InterAg, Hamilton, New Zealand) for nine days and a double intramuscular (i.m.) injection of a synthetic prostaglandin (Lutalyse, 5 mg/ewe, Pharmacia & Upjohn, UK), 12 h before and at removal, to ensure adequate corpus luteum regression. The time of progesterone withdrawal (i.e. the commencement of the follicular phase) is referred to hereafter as time 0 h. Animals treated with insulin (n=24; neutral zinc bovine insulin, Hypurin Neutral, CP Pharmaceuticals, Wrexham, UK) received an i.v. dose of 4 iu/kg body weight at 28 h and 30 h after progesterone withdrawal and those treated with endotoxin (n=12; Lipopolysaccharides Escherichia coli 055:B5, Sigma-Aldrich, UK; LPS) were given an i.v. dose of 100 ng/kg body weight at 28 h. Controls (n=24) received 2 ml of saline vehicle. The dose of LPS administered was lower than that used by others (Battaglia *et al*., 2000) to avoid overt clinical responses. In a preliminary trial, 6 ewes were given either 50 or 100 ng/kg body weight LPS and we concluded that 100 ng/kg provided a robust cortisol response and a delayed LH surge, with minimal clinical signs of occasional coughing. In the main study, a total of 29 blood samples per ewe were collected at time 0 h, 10 h, 14h, 20 h and subsequently at 2 h intervals till 80 h. Core body temperature was evaluated per rectum with an electronic thermometre. Two control measurements were obtained from all animals at 0 h then every 2 h for a period of 12 h after LPS administration.

The frequent blood sampling, as well as the administration of all substances, was facilitated by the insertion of a silastic catheter into a jugular vein of each ewe under local anesthesia before progesterone withdrawal. Patency was maintained with heparinised saline (Multihep, 100 iu/ml, Leo Laboratories, Princes Risborough, UK)

administered after each blood withdrawal. Blood samples were collected from all ewes into heparinised glass tubes and centrifuged immediately at 1000 *g* for 20 min at 4^0C. Plasma was stored at -20^0C until analysis.

Plasma analysis

Samples, in duplicate, were analysed by a second antibody Enzyme-Linked Immunosorbent Assay (ELISA) for LH, and by single antibody ELISA for pregnane metabolites (equivalent to and hereafter referred to as progesterone) or cortisol. These assays were performed with methods adapted from Graham *et al.* (2002), Munro and Stabenfeldt (1984) and Young *et al.,* (2004), respectively. LH results were expressed as ng equivalent of NIAMDD ovine LH 21 per ml plasma. Oestradiol was measured with a modified radioimmunoassay (RIA) described by Walker *et al.* (2008) using 0.5 ml plasma sample extracted with 3 ml diethyl ether followed by evaporation to dryness. 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 with an antibody dilution characterised and verified in our laboratory. In general, 100 µl aliquots of plasma were assayed initially, and samples that were over the maximum standard were re-assayed after a ten-fold or fifteen-fold dilution. Plasma glucose and triglycerides were measured by the hexokinase/glucose 6 phosphate dehydrogenase and lipase/glycerol kinase methods, respectively, using commercial kits (Boehringer Mannheim, Lewes, Sussex) on a Konelab 20i autoanalyser (Thermo Electron Corporation). The inter-assay coefficient of variation was less than 10% for samples within the range of 1 to 4 mmol/l and 0.1 to 0.4 mmol/l for glucose and triglycerides, respectively. Analysis of glucose, triglycerides, cortisol and progesterone, was restricted to samples taken till 44 h after progesterone withdrawal, whereas LH was measured in all samples and oestradiol was restricted to samples taken at 0 h, 10 h, 30 h and then every other sample till the end of the LH surge.

Visual observation of oestrous behaviour

Ewe and ram oestrous behaviour was monitored by two trained observers for a 30-minute observation period prior to each blood sample collection. Paint spray had been used to place a large symbol on the back and sides of each ewe and ram. The observers were elevated 1.5 m above the pen so identification of symbols and all activities could be seen without disturbing the animals. At each observation period, quantitative and qualitative data were recorded for each ewe and ram individually. Retrospectively, the frequency of each behaviour was calculated. Once a minute throughout each observation period, it was noted if a ewe was within one metre of a ram (behavioural scan sampling; Martin and Bateson (1986). In addition, frequencies of the following behavioural signs of oestrus were noted throughout each 30 min observation period: perineal region of a ewe being nosed by a ram; tail fanning by a ewe (each bout of swift side-to-side tail movements); ewe being nudged by a ram without the ewe moving away; and, subsequent mounting of the ewe by a ram without the ewe moving away. Due to the 2-hourly observation regime, the beginning or end of a period was respectively defined as the first (minus 1.0 h) or last (plus 1.0 h) 30-min observation period the animal exhibited a particular behavioural sign; duration was taken as the time between the beginning and end. Behaviour onsets were considered as the first time that the behaviour was observed, when closely followed or accompanied by other behaviours.

Data analysis

An LH surge was defined as a sustained increase in LH plasma concentrations (above 15 ng/ml for 3 consecutive samples taken every two hours) and it was considered to begin (surge onset) and end when the first value increased or decreased, respectively, more than five times the assay minimum detectable quantity (i.e. > 5 ng/ml). Hormone and behaviour data were analysed with Minitab® 15 statistical package (MINITAB Inc, Pennsylvania, USA). For all analyses, statistical significance was considered when $P<0.05$. Data were tested for normality with the Anderson-Darling test. Most of the data were not normally distributed, even after log transformation; therefore, non-parametric statistical analysis was used throughout (Kruskal-Wallis and Mann Whitney tests, as well as Wilcoxon signed rank test for pair wise comparisons). Hormone data were compared

between groups at each time point unless otherwise stated. Intervals from progesterone withdrawal to first onset of each behaviour or the LH surge between groups, and between the onsets of each behaviour or the LH surge within a group were compared by Kaplan-Meier survival analysis (non-parametric distribution analysis). Regression analysis was used to determine the association between the duration of oestradiol decrease and the onset of the LH surge. Results are expressed as mean ± SEM.

Results

Two animals from each of the control, insulin and LPS groups exhibited signs of oestrus and were mounted by a ram within 28 h after progesterone withdrawal (i.e. before the predetermined time of treatment), whereas one insulin-treated animal did not respond to the synchronization protocol with a decrease in progesterone values. Thus, the data from these 7 ewes were excluded from further analysis. Treated animals did not show any signs of illness, with a few exceptions of mild coughing and increased respiration rate for the ewes that received LPS.

Endocrine profiles: LH, cortisol, progesterone and oestradiol

All animals had an LH surge regardless of treatment. A delayed LH surge onset was defined as any time greater than the median onset in the control animals plus one inter-quartile range. The control animals had a median onset at 35.0 h after progesterone withdrawal, with an inter-quartile range of 9.5 h; hence, animals classified as 'delayed' had an onset later than 44.5 h after progesterone withdrawal. During data analysis, it became clear that the insulin group consisted of two subgroups, referred to hereafter as insulin-delayed or insulin-non-delayed.

As shown in Table 1, there was a delay to the time of LH surge onset comparing the controls with insulin-delayed subgroup (17.6 h delay; $P<0.001$) or LPS group (22.5 h delay; $P<0.001$), as well as an extra 4.9 h delay ($P<0.05$) for the LPS group compared to the insulin-delayed subgroup. However, surge durations did not differ between groups ($P=0.7$). The LH surge amplitude was lower in the insulin-delayed subgroup compared to the control group (73.4 ± 5.2 h *vs* 46.0 ± 2.3 ng/ml; $P<0.05$) but amplitudes for the LPS group were not different from controls (Table 1).

In all control animals, the concentration of cortisol remained between 7.1 to 15.2 ng/ml throughout the study; and before the application of insulin or LPS, cortisol values in all groups were not different (Fig. 1). Cortisol increased after LPS and insulin administration (P<0.05; Fig. 1) with maximum values of 100.6 ± 17.3 ng/ml, 108.7 ± 14.2 ng/ml and 186.8 ± 9.2 ng/ml (for insulin non-delayed, insulin-delayed and LPS groups, respectively) 4 h after the first injection. Concentrations remained elevated for 12 h after treatment compared to controls (Fig. 1). The LPS group had higher concentrations compared to the insulin subgroups at 30 h, 32 h and 34 h (P<0.05 for all comparisons; Fig. 1). Cortisol values in the two insulin subgroups did not differ at any time (Fig. 1).

Progesterone concentrations at 0 h and 28 h after progesterone withdrawal were not different between all groups (28.0 ± 3.1 ng/ml and 4.6 ± 0.6 ng/ml, respectively). Thereafter, due to large between-animal variation, a within-animal comparison was made. Progesterone concentrations were different in the insulin-delayed and LPS groups, increasing from 4.0±0.5 ng/ml and 5.3±1.0 ng/ml to a mean maximum of 5.7±0.3 ng/ml and 8.8±1.6 ng/ml, respectively (P<0.05 for both comparisons; Fig. 2). Control and insulin-non-delayed groups had similar concentrations of progesterone before and after treatment (Fig.2).

Oestradiol concentrations between all groups were not different up to 28 h after progesterone withdrawal. Thereafter, oestradiol profiles varied considerably (see examples in Fig. 3). Thus, to evaluate the effect of insulin or LPS on oestradiol concentrations, the percentage change between 30 h and the average of the 2 lowest values, after treatment but before LH surge onset, as well as the duration of decrease, was calculated and compared between groups (Fig. 4). In control animals, oestradiol concentrations continued to increase after saline (Fig. 4), whereas in the insulin-non-delayed animals two types of hormone profiles were observed, with oestradiol in some ewes appearing to be unaffected by treatment (Fig. 3; ewe Q), and others exhibiting a sudden decrease in concentrations followed by a quick recovery (Fig. 3; ewe W). Oestradiol concentrations in the insulin-delayed and LPS groups began to decrease at 30 h after progesterone withdrawal (i.e. 2 h after the time of treatment), exhibited a 50% and 40% reduction, respectively (P<0.01 for both; Fig. 4) and then returned to pre-treatment values at 46.2 ± 1.4 h (insulin-delayed) and 52.0 ± 2.1 h (LPS) after

progesterone withdrawal. Thus, the duration of lowered oestradiol values was 5.1 h shorter in the insulin-delayed subgroup compared to the LPS group (16.2 ± 1.3 *vs* 21.3 ± 1.9 h; *P*<0.05; Fig. 4). Furthermore, onset of the LH surge was associated with the duration of decreased oestradiol values (*P*<0.001; Rs-q= 78%). The LH surge onset in these two groups, occurred 7.5 ± 1.4 h after oestradiol concentrations returned to pre-treatment values.

Core body temperature

Rectal temperatures were not different between all animals prior treatment (38.7 ± 0.1 ^{0}C). After LPS treatment, there was an increase to a maximum of 40.7 ± 0.1 ^{0}C four hours after treatment (38.7 ± 0.1 ^{0}C vs. 40.1 ± 0.1 ^{0}C, P<0.001; Fig. 5), in parallel with cortisol concentrations.

Glucose

In all animals, the concentration of glucose remained between 2.7 to 3.1 mmol/l before treatment. Values in control animals were not different before and after saline (3.1 ± 0.1 mmol/l vs. 3.0 ± 0.1 mmol/l; respectively). In contrast, insulin-non-delayed, insulin-delayed and LPS concentrations of glucose decreased within 2 h of the first injection then returned to pretreatment values by 40h and 42 h (for the insulin-non-delayed and insulin-delayed subgroups, respectively; Fig. 6, panel A) and 56 h (for the LPS group; Fig. 6, panel B) after progesterone withdrawal. Glucose concentrations were not different between insulin-non-delayed and insulin-delayed subgroups throughout the study (Fig 6, panel A).

Triglycerides

Triglyceride concentrations were not different between the insulin-non-delayed and insulin-delayed subgroups as indicated by five measurements between 0 and 34 h after progesterone withdrawal (Fig. 7 insert). Insulin treatment did not affect plasma concentrations (before and after; 0.25 ± 0.01 mmol/l versus 0.21 ± 0.01 and 0.25 ± 0.01 mmol/l versus 0.26 ± 0.01 mmol/l in the insulin-non delayed and insulin delayed subgroups, respectively; Fig. 7).

Behavioural profiles

All animals displayed signs of oestrus behaviour during the study. Figure 8 shows the mean (± SEM) onset and duration of individual oestrus behavioural signs in control, insulin and LPS-treated animals. In control animals, pre-copulatory behaviour began 29.2 ± 1.3 h after progesterone withdrawal with the ram and ewe moving near to each other and this state lasted longer than all other behaviours (P=0.006; Fig. 8). At 31.5 ± 1.2 h (2.3 h later; P<0.05), the rams began nosing the ewes' perineal region and this lasted longer than subsequent behaviours (P=0.002; Fig. 8). The onset of being nosed was followed 6 h later by the simultaneous expression of tail fanning by the ewe, being nudged and mounted by a ram (at 37.5 ± 1.2 h after progesterone withdrawal; P<0.05). Durations of tail fanning and nudging were not different, whereas mounting lasted for approximately 10 h and was thus the behaviour with the minimum duration (P=0.02; Fig. 8) and frequency (7.7 ± 0.9 mounts throughout the study; P=0.002). Being nosed was the most frequent and consistently observed behavioural sign between animals, with an average of 20.5 ± 1.4 nosing events in the controls throughout the study (P<0.05). Being nudged was also more frequent than being mounted (12.6 ± 1.3 nudges *vs* 7.7 ± 0.9 mounts; P=0.004).

Comparison of behaviour profiles between treatment groups especially with respect to the LH surge onset

The onset, duration and total frequency of each behaviour sign was disturbed by treatment within each group as well as between treatment groups (Fig. 8 and Table 2). Onsets of all behaviours were delayed in the insulin-delayed (P<0.05, for all) and LPS (P<0.001, for all) groups but not in the insulin-non-delayed subgroup (P>0.5; Fig. 8). The duration of expression of all behaviours was not different between control and treated groups, apart from ewes being near the ram(s) which was shorter in the LPS group (P<0.05; Fig. 8). The frequency of being mounted was decreased in the LPS group (P=0.009 and tended to increase in the insulin-delayed subgroup; P=0.06). The total frequency of all other behaviours (i.e. minutes spent near ram(s), being nosed, tail fanning and being nudged) was not different between groups (Table 2). The interval from the onset of each behaviour and the LH surge was longer in the insulin-delayed subgroup compared to all other groups (treated and control; Fig. 8 and Fig. 9).

Discussion

Responses to the administration of insulin resulted in two groups: the timing of pre-ovulatory behaviour and the LH surge was not affected in half the ewes but, in the other half, the onset of behaviour was delayed by ~7 h and the LH surge by ~17 h. This explains the large variation in data from our smaller insulin study (Saifullizam *et al,* 2010*).* In contrast, LPS delayed both the onsets of behaviour and the LH surge by ~22 h. The marked ~10 h disparity between the onsets of oestrus and the LH surge in half the insulin-treated ewes concurs with our recent findings that acute stress with insulin separated the onsets of behaviour and the LH surge in some intact ewes (Saifullizam *et al.,* 2010). However, we now extend these observations by reporting that LPS does not have the same effect, with both components being equally delayed, suggesting that the dose of LPS used is a 'stronger' stressor than that of insulin, or the two stressors act via different pathway(s) to alter reproductive parameters.

In ruminants, oestradiol is considered as the primary "trigger" for the onset of behavioural and GnRH/LH surge events (Fabre-Nys *et al.,* 1994, Fabre-Nys and Gelez 2007) although recent reports suggest that the threshold concentration for the induction of oestrus behaviour is lower than that for the induction of the GnRH/LH surge (Ben Said *et al.,* 2007, Saifullizam *et al.,* 2010) and therefore the triggering signals may be different (Caraty *et al.,* 2002). In the present study, oestradiol concentrations decreased after treatment, and returned to pretreatment values after ~17 h in the insulin-delayed subgroup and ~22 h in the LPS group (i.e. similar times that the surges were delayed). Furthermore, both groups had an LH surge onset ~ 7 h after restoration of oestradiol concentrations. We, therefore, hypothesise that when a stressor is applied during the late follicular phase, the duration of the LH surge delay is related to the duration of oestradiol signal disruption caused by these stressors. Furthermore, the present findings support existing evidence that for the induction of a GnRH surge, it is not only important for a threshold of oestradiol concentration to be reached, but also a specific duration of high concentration is required (Caraty *et al.,* 2002, Ben Said *et al.,* 2007).

Interestingly, in the LPS group, all sexual behaviours occurred at similar times relative to the LH surge as in controls, whereas in the insulin-delayed subgroup these behaviours began before oestradiol returned to pretreatment values and with a 10 h disparity. Thus, insulin-treated animals were able to overcome the inhibition of the behaviour generating mechanism, quicker than the inhibition of the GnRH surge generating mechanism. We propose three possible explanations for this disparity. First, the insulin-delayed and LPS groups had an increase in progesterone concentrations and this subtle change may have been the mechanism by which all sexual behaviours were blocked in these groups (Scaramuzzi et al., 1971, Fabre-Nys, 1998, Fabre-Nys and Gelez 2007). When progesterone concentrations decreased and oestradiol reached the appropriate threshold, sexual behaviour began. The shorter delay observed in the insulin-delayed group, could be due to the fact that the increase in progesterone was less intense than in the LPS group. Second, although not yet confirmed, the behaviour generating mechanism may be comprised of three phases, similar to those of the GnRH mechanism (activation, transmission, and surge secretion; Evans *et al.,* 1997, Harris *et al.,* 1998, Harris *et al.,* 1999). LPS may have blocked any of those phases for longer compared to insulin, via an unknown mechanism. Third, in the present study, maximum LH values achieved during the surge were lower after insulin but not LPS, indicating that pituitary responsiveness to GnRH may have been compromised (Smith *et al.,* 2003). Therefore, it is possible that in insulin-treated animals, GnRH pulses were restored before the pituitary regained sensitivity to GnRH and therefore behaviour was driven centrally by those pulses (Caraty *et al.,* 1998, Caraty *et al.,* 2002). Simultaneous GnRH/LH pulse and oestrus monitoring could help to elucidate this point. Interestingly, Brothers *et al.,* (2010) report a profound insulin sensitivity of the pituitary gland that outlasts hypothalamic effects and this may be a reason for a disruption of the one-to-one relationship between GnRH and LH pulses.

Progesterone withdrawal plays a major role in synchronizing physiological and behavioural events (Fabre-Nys and Martin 1991). In our study, all animals were primed with progesterone (both endogenously and via the applied CIDRs) and had similar concentrations at 0 h (CIDR withdrawal) and at 28 h (time of treatment). Progesterone has the ability to inhibit the LH surge when applied during the activation and/or

transmission phases (Richter *et al.,* 2002, Smith *et al.,* 2003, Richter *et al*., 2005). However, the dose of progesterone used in those experiments, was provided by two vaginal pessaries (CIDRs) and the artificial increase in peripheral concentrations were equivalent to luteal phase concentrations. In our studies, the increase in progesterone concentration in the insulin-delayed and LPS treated animals was much smaller than in the above studies. Furthermore, maximum progesterone concentrations after treatment occurred at variable times, but always after oestradiol had started to decrease. Therefore, it is unlikely that the progesterone increment is the sole mediator for lowering oestradiol concentrations and delaying the LH surge. In support of this, the insulin-induced LH surge delay is not reversed by administration of the progestin/glucorticoid receptor antagonist RU486 (Dobson and Smith 2000).

Despite extensive investigation, there continues to be a debate whether glucocorticoids play a direct role in suppressing reproductive neuroendocrine function in response to stress. In the present study, cortisol values increased in all treated groups for a 12-h period after treatment, reaching higher values for ~ 4 h in the LPS group compared to insulin subgroups. Recent evidence in ovariectomised (OVX) ewes suggests that the ability of cortisol to alter LH pulse frequency (Oakley *et al.,* 2009) as well as pituitary responsiveness to GnRH (Pierce *et al.,* 2009), depends on the presence of follicular phase concentrations of oestradiol. However, in the present study, as soon as the stressors were applied and cortisol values began to increase, there was a concurrent sharp decrease in oestradiol concentrations. Furthermore, cortisol increased equally in both delayed and non-delayed insulin treated animals. Therefore, even in the presence of oestradiol (there was no oestradiol decrease in the insulin-non-delayed subgroup even though cortisol increased) increased cortisol was not associated with delays in the LH surge. This leads us to believe that, in this 'more natural' follicular phase ewe model, in which ovarian steroid production, feedback mechanisms and stressor-induced mediator hormones were all present, cortisol was not the key disruptor of the LH surge.

Interestingly, 50% of the animals treated with insulin did not have a delay in sexual behaviour or the LH surge (insulin-non-delayed subgroup). Furthermore, both insulin groups had similar cortisol and glucose plasma profiles throughout the study. Insulin-non-delayed ewes had two types of oestradiol profiles, with some ewes appearing to be

unaffected by treatment, whilst others showed a sudden decrease in concentrations followed by a quick recovery. Therefore, the H-P-A axis in this group was activated without altering any reproductive parameters. So, why did this split response occur? One might argue that, in these sheep, the activation phase occurred before treatment, but insulin is known to delay the LH surge when applied in any phase of the GnRH generation mechanism (Smith *et al.,* 2003, Medina *et al.*, 1998). In fact, injection of insulin before or even up to 16 h after the insertion of subcutaneous oestradiol implants in OVX ewes delayed the LH surge (Medina *et al.,* 1998). Furthermore, if the above was true then a similar split response would have been observed in the LPS treated animals. We tested whether insulin resistance was involved (Kaske *et al.,* 2001), by measuring triglycerides, but again there was no difference between groups. Further studies of brain tissue obtained from these sheep may clarify this point.

Beach (1976) proposed a classification of female sexual behaviour into three components: attractivity, proceptivity and receptivity. However, the sexual behaviours that can be observed at farm level are not all easily classified into one of the above categories. We measured sexual behaviour in an open-field situation (Saifullizam *et al.,* 2010), and recorded their sequential appearance: ~29 h after progesterone withdrawal the ewe and ram chose to be within 1m of each other, at ~32 h the ram(s) started to nose the ewes perineum and at ~38 h the ewe was tail fanning, nudged and mounted by the ram(s). The fact that all behaviours were delayed together as a group, whilst durations and frequencies did not differ, suggests that they all have a common regulating factor, presumably oestradiol (Fabre-Nys and Gelez 2007). However, the fact that they begin and end sequentially implies that they also have independent controlling mechanisms. In support of this, Pierce *et al*. (2008) used a layered paradigm of psychosocial stressors and reported a reduction in the intensity of attractivity and proceptivity but not receptivity. However, a recent report by Papargiris *et al.* (2011) noted that cortisol was able to suppress receptivity but not attractivity or proceptivity presumably by interfering with the action of oestradiol to induce receptivity. Furthermore, feed-restriction in female voles was followed by a decrease in circulating oestradiol concentrations and a reduction or suppression of attractivity, proceptivity, and receptivity, however, oestradiol treatment was not sufficient to restore attractivity

(Pierce *et al.,* 2007). All these observations concur that the stress-induced suppression of sexual behaviour cannot be attributed to a single factor, but is rather multi-factorial. In this aspect, it has been reported that in many species, pheromones serve as sexual attractants, promote sexual arousal and mating behaviour as well as modifying neuroendocrinological aspects of reproduction (Johnston and Bronson, 1982; Okamura et al. 2010). However, their precise role in exhibition of pre-copulatory behaviours or the effects of stressors on their production remain to be elucidated.

Successful reproduction requires follicular maturation and oestradiol biosynthesis, induction of the LH surge, ovulation, and expression of sexual behaviour to be co-ordinated within a tight time-frame. However, the LH surge onset and the onset of oestrus were delayed by ~17 h and ~7 h, respectively, after acute insulin administration and ~ 22 h and ~ 21 h, respectively, after acute LPS administration. Oestradiol concentrations decreased after treatment, and remained low for a period equivalent to the LH surge delay. We, therefore, hypothesise that when a stressor is applied during the late follicular phase, the duration of the LH surge delay is related to the duration of oestradiol signal disruption. Interestingly, 50% of the animals treated with insulin did not display a delay in sexual behaviour or the LH surge, even though cortisol increased and glucose decreased after treatment, in an identical fashion to the delayed ewes. Even though the reasons for this split response are not yet clear, this unexpected finding provides direct evidence that cortisol is not the key disruptor of either the LH surge or sexual behaviour after insulin administration in intact ewes. The fact that insulin-treated animals were able to overcome the inhibition of the behaviour generating mechanism quicker than the inhibition of the GnRH surge generating mechanism by 10 h provides a model that can be used to identify the specific neuronal systems that control behaviour distinct from those initiating the GnRH surge.

Acknowledgements

Thanks are due to Nigel Jones and the farm staff for care of the animals; Hilary Purcell, David Jones and Peter Taylor for technical assistance; and Prof A Parlow and NIAMDD, USA for LH standard preparations. We are also grateful to Dr. Lucy Pickavance for many useful suggestions.

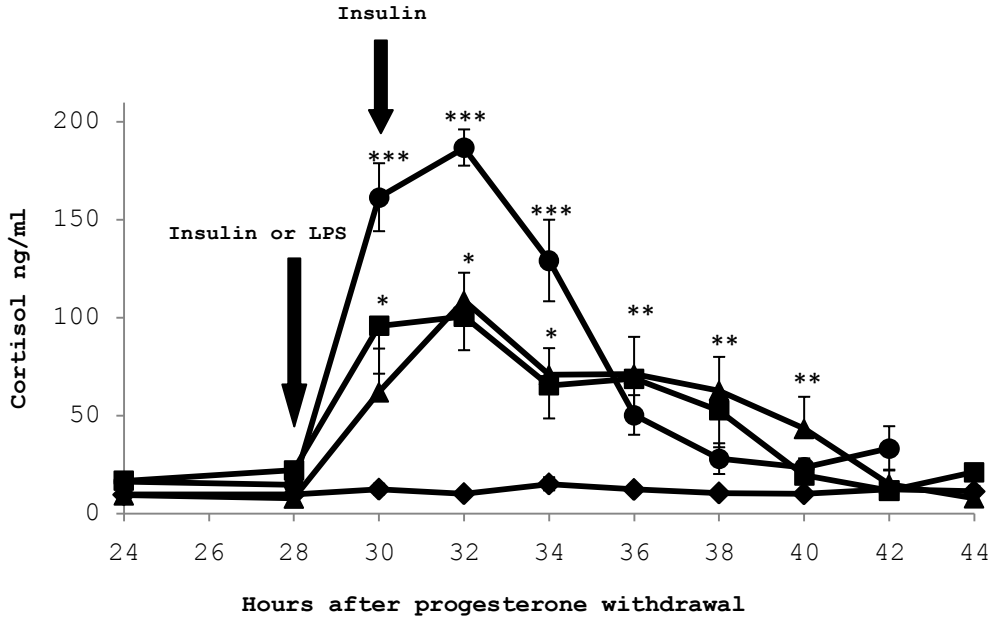

Fig. 1 Mean (± SEM) plasma cortisol concentrations in the control (♦; n=22), insulin-non-delayed (■; n=10), insulin-delayed (▲; n=11) and LPS (●; n=10) treated ewes. The arrows indicate the time of treatment. Some error bars are within the data symbol.

***** Times at which both insulin subgroups were different from controls (P<0.05).

****** Times at which treated groups were different from controls (P<0.05).

******* Times at which the LPS cortisol values were different from the control group (P<0.001) and the insulin subgroups (P<0.05).

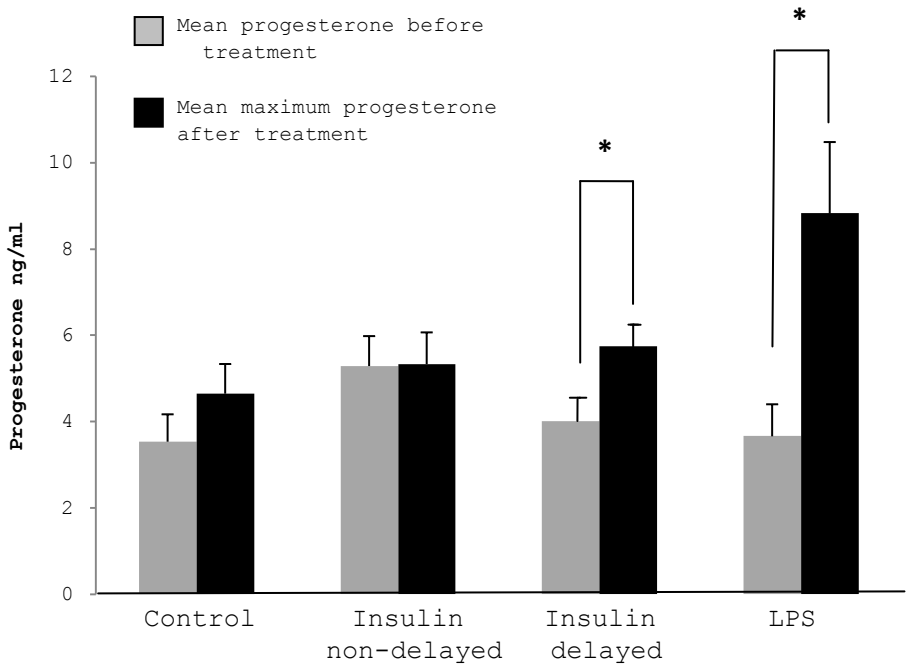

Fig. 2 Mean (± SEM) plasma progesterone concentrations within an animal before treatment (grey bars) and mean (± SEM) maximum concentrations after treatment (black bars), in 22 control, 21 insulin treated (10 insulin-non-delayed and 11 insulin-delayed) and 10 LPS treated ewes. The differences between concentrations within an animal are linked by the line (* $P<0.05$).

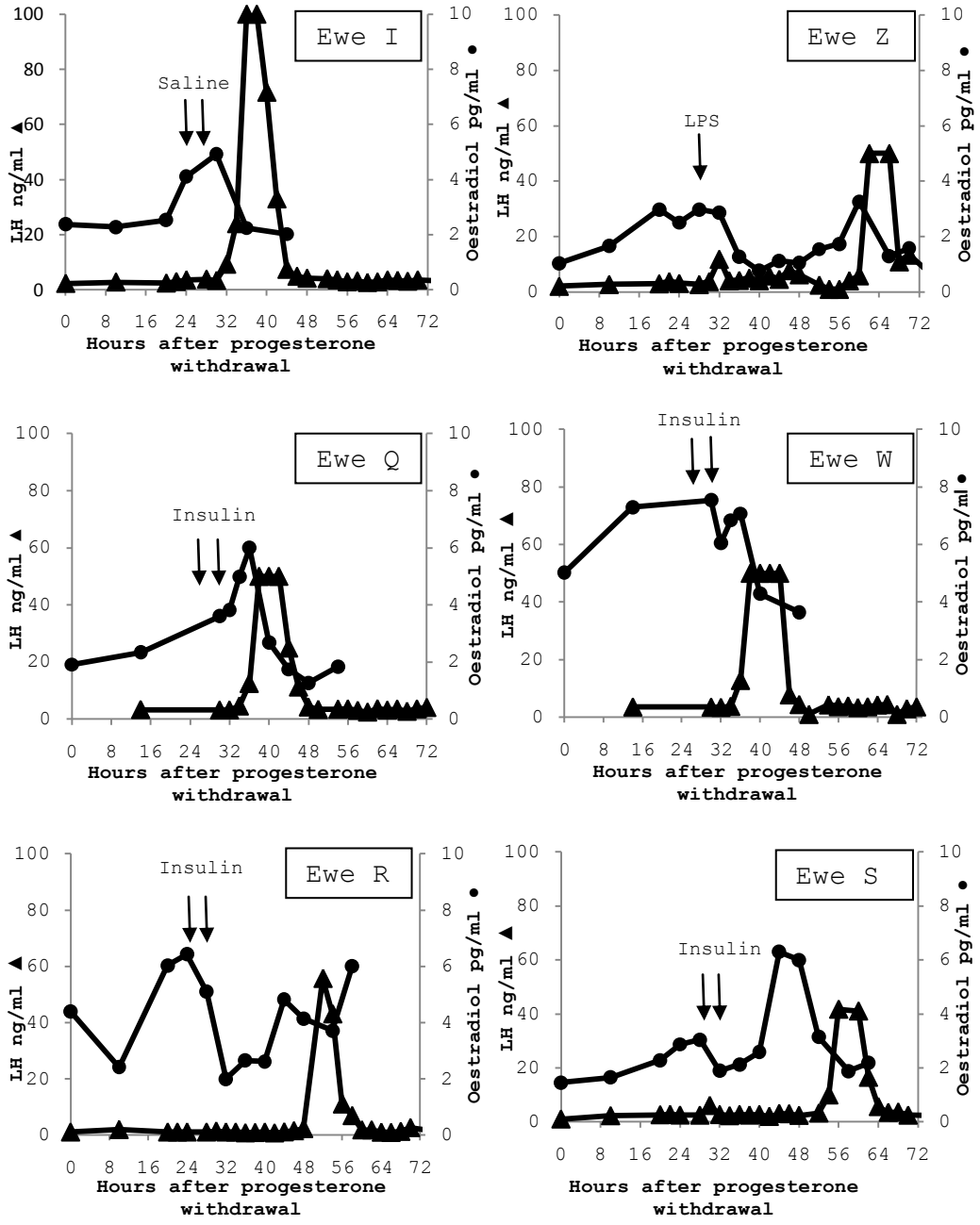

Fig. 3 Representative individual oestradiol (●) and LH (▲) hormone profiles in control (ewe I), LPS (ewe Z), insulin-non-delayed (ewe Q and W) and insulin-delayed (ewe R and S) treated ewes relative to progesterone withdrawal (time 0 h). Arrows indicate the time of treatment.

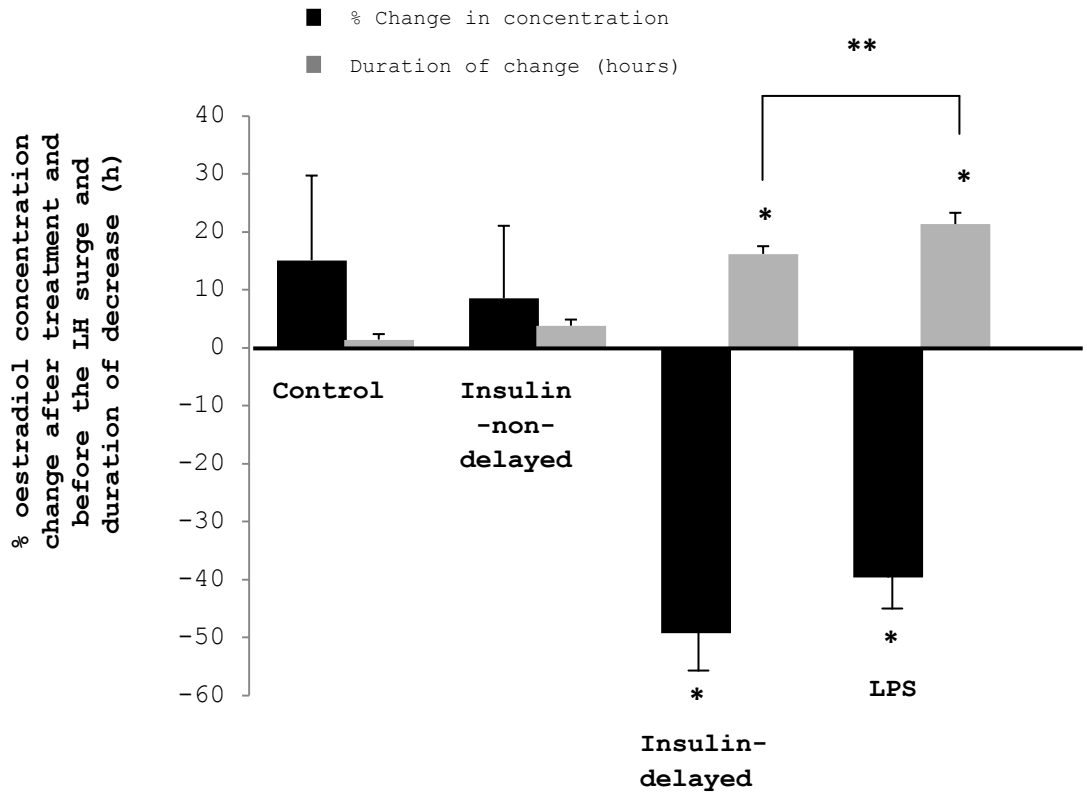

Fig. 4 Change of oestradiol concentration between 28 h (i.e. the predetermined time of treatment) and the 2 lowest values recorded after treatment but before LH surge onset (black bars) and duration of decrease (grey bars), in control (n=22), insulin (non-delayed n=10, delayed n=11) and LPS (n=10) treated ewes.

* *P*<0. 01 compared to control and insulin-non-delayed groups; *P*<0.05 difference in duration indicated by line and **.

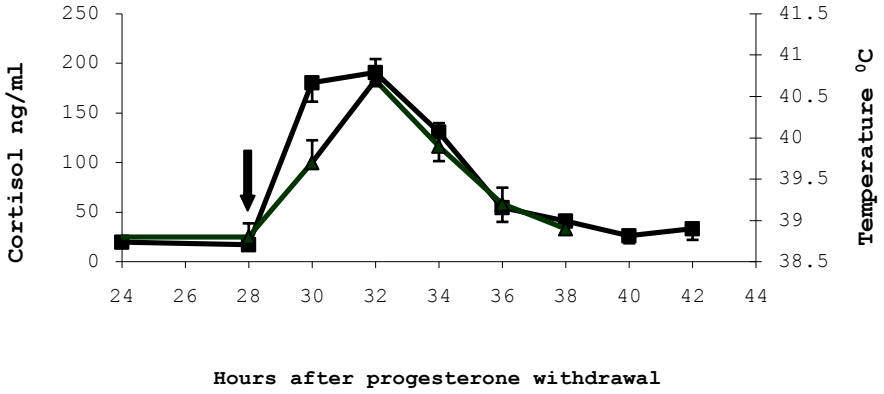

Fig. 5 Mean (± SEM) plasma cortisol (■) and rectal temperature (▲) responses to LPS In 10 ewes: rectal temperature increased by 1.8^{0}C. The arrow indicates the time of treatment. Some error bars not visible are within the data symbol.

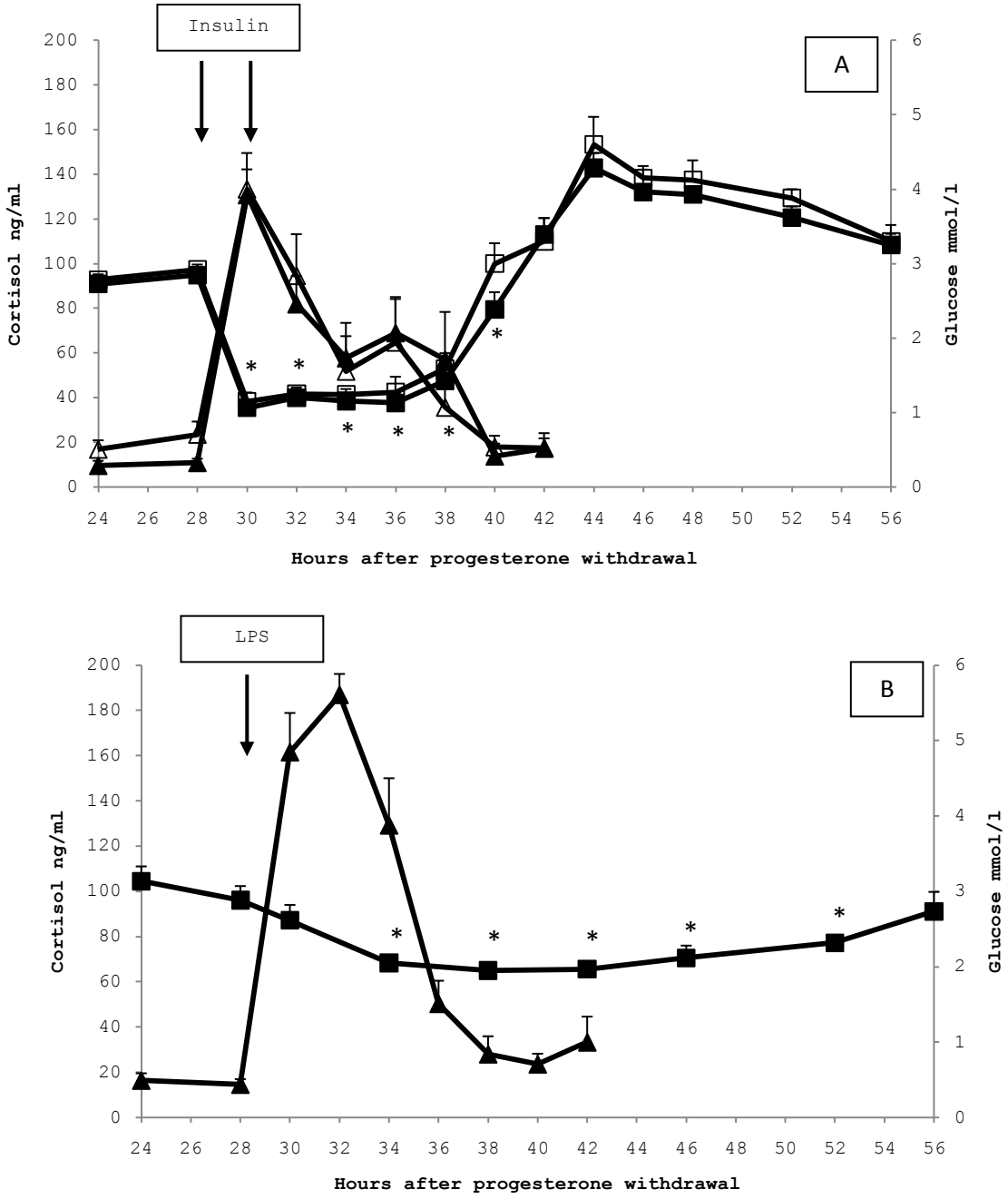

Fig. 6 Mean (± SEM) plasma cortisol (▲) and glucose (■) in the insulin-non-delayed (n=10; open symbols, panel A), insulin-delayed (n=11; closed symbols, panel A) and LPS (n=10; closed symbols, panel B) groups, after two injections of insulin (4 iu/kg) or a single injection of LPS (100 ng/kg). The arrows indicate the time of treatment. Some error bars that are not visible are within the data symbol. * P<0.05 compared to glucose concentrations at 28h after progesterone withdrawal (i.e., the time of treatment).

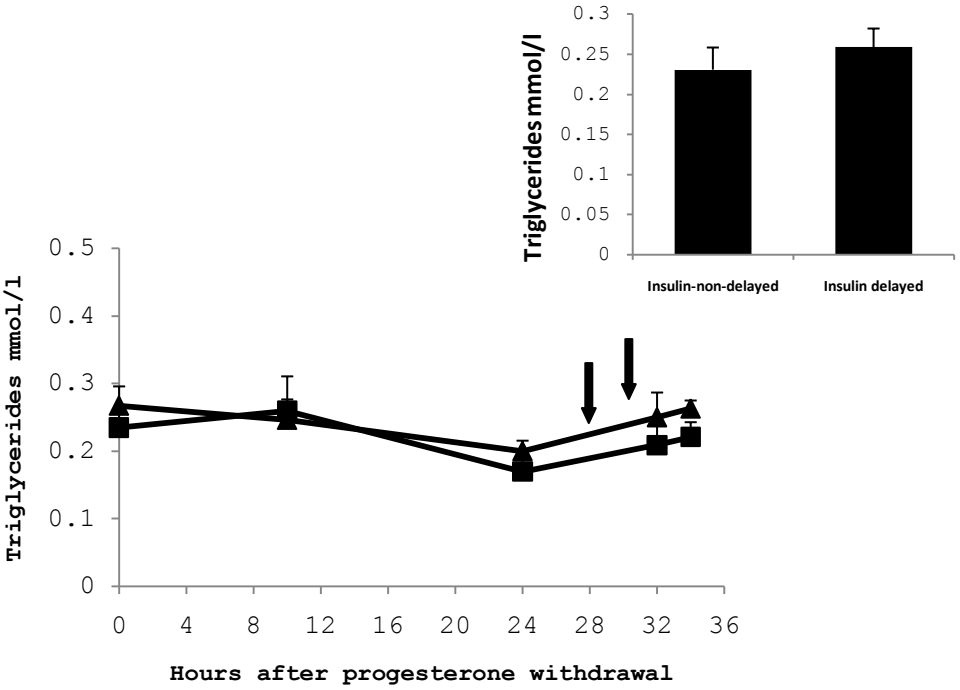

Fig. 7 Mean (± SEM) plasma triglyceride concentrations of the insulin-non-delayed (■; n=10), insulin-delayed (▲; n=11) subgroups from 0 to 34 h after progesterone withdrawal. The arrows indicate the time of treatment. Some error bars not visible are within the data symbol. **Insert:** Overall group mean (± SEM) of individual ewe mean plasma triglyceride concentrations after 5 measurements per ewe within a 34 h time period.

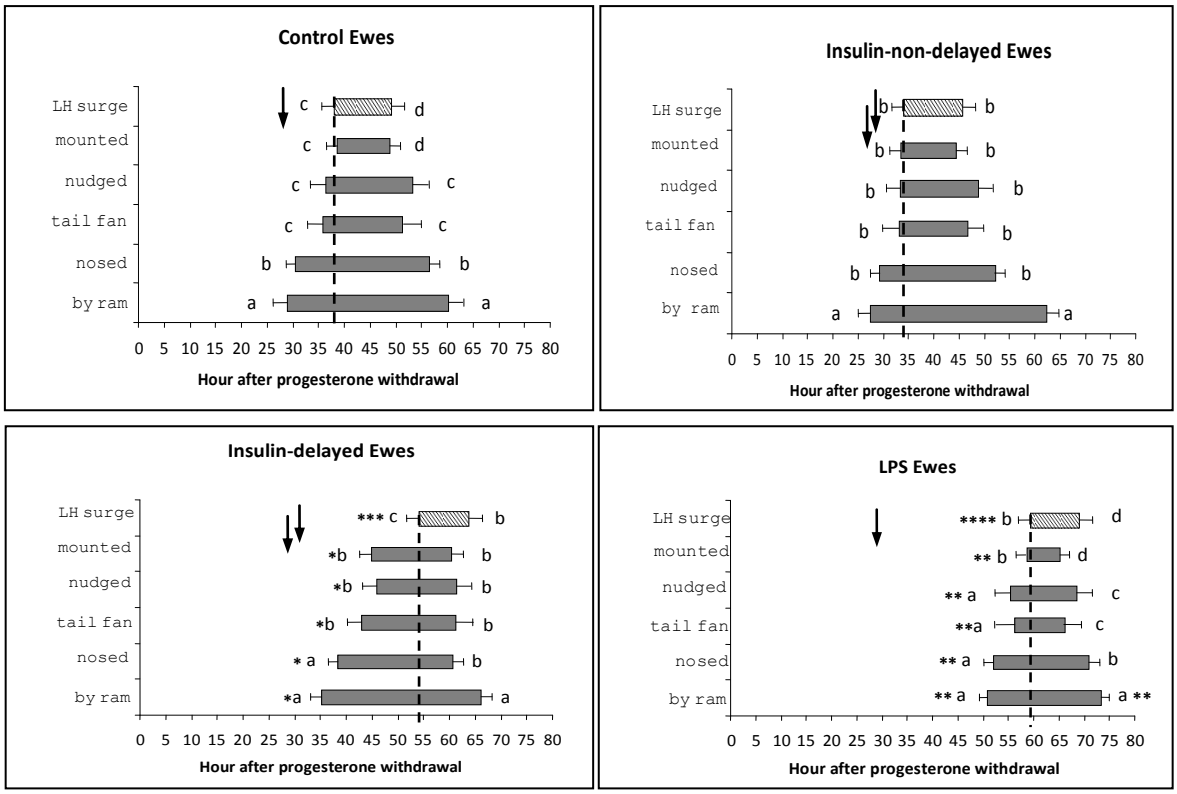

Fig. 8 Mean (± SEM) hours from first to last display of different oestrus behaviours after progesterone withdrawal in 22 control ewes, 21 ewes injected with 4 iu/kg insulin (non-delayed n=10, delayed n=11) at 28 and 30 h, and 10 ewes injected with 100 ng/kg LPS at 28 h after progesterone withdrawal. Timing of the LH surge is also shown. Within each panel, differences between the onset of each behaviour are indicated by different letters at each end of each bar, respectively (P<0.05); differences between the duration of each behaviour are also indicated by the letters at the end of each bar (P<0.02). Differences in the timing of onset between panels are indicated with asterisks. Time of treatment is indicated with the arrows. * P<0.05 compared to controls and insulin-non-delayed groups, **P<0.001 compared to control and both insulin groups, *** P<0.001 compared to controls and insulin-non-delayed groups. **** P<0.05 compared to controls and insulin subgroups.

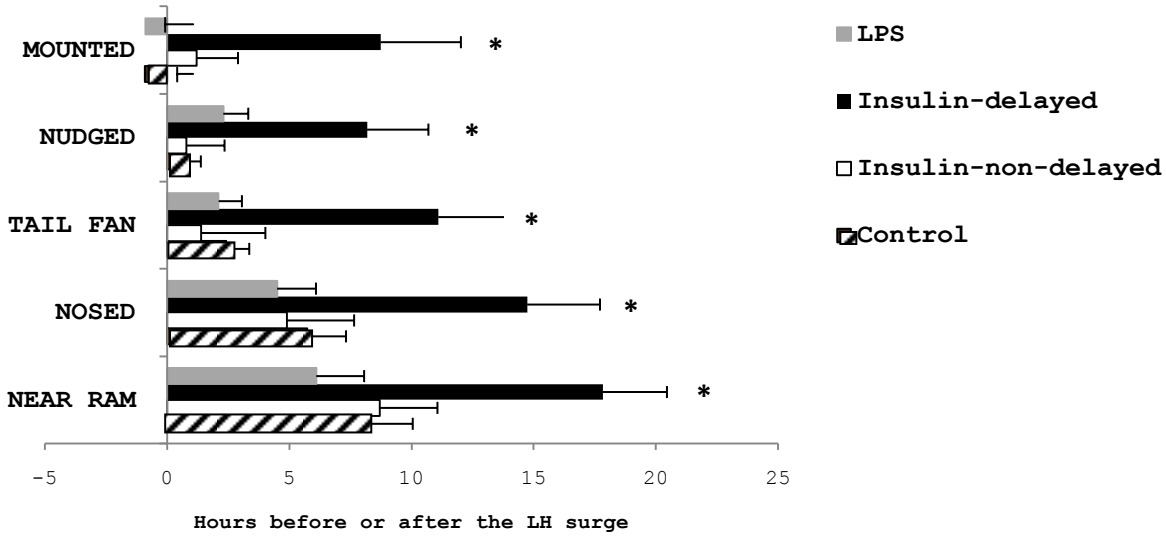

Fig. 9 Mean (± SEM) interval from the onset of individual behaviours to the LH surge onset (time 0 h on the graph) in 22 control, 21 ewes injected with 4 iu/kg insulin (non-delayed n=10, delayed n=11) at 28 and 30 h, and 10 ewes injected with 100 ng/kg LPS at 28 h after progesterone withdrawal. * P<0.03 compared to all behaviours between all groups.

Table 1 Effect of saline, insulin or LPS on LH surge parameters in 22 control, 21 ewes injected with 4 iu/kg insulin (non-delayed n=10, delayed n=11) at 28 and 30 h, and 10 ewes injected with 100 ng/kg LPS at 28 h after progesterone withdrawal.

	Controls	Insulin-non-delayed	Insulin-delayed	LPS
N [a]	1/22	0/10	11/11	10/10
Incidence (%)	100	100	100	100
Onset (h [b]**)**	36.5 ± 1.2	34.5 ± 0.7	54.1 ± 1.4[d]	59.0 ± 1.7[e]
Duration (h)	12.2 ± 0.5	12.0 ± 0.8	11.4 ± 0.9	11.6 ± 0.5
Amplitude (ng/ml)	73.4 ± 5.2	60.7 ± 9.2	46.0 ± 2.3[c]	72.2 ± 11.1

[a] N = number of animals within each group that had a delayed LH surge onset.

[b] h = hours after progesterone withdrawal.

[c] Within a row $P<0.05$ different from control group values.

[d] Within a row $P<0.05$ compared to the control, insulin-non-delayed group.

[e] Within a row $P<0.05$ compared to the control and insulin subgroups.

Table 2 Mean (± SEM) of total frequencies of different behaviours (being nosed, tail fanning, being nudged and being mounted) displayed per ewe during the whole oestrus period in 22 control, 21 ewes injected with 4 iu/kg insulin (non-delayed n=10, delayed n=11) at 28 and 30 h and 10 ewes injected with 100 ng/kg LPS at 28 h after progesterone withdrawal.

	Controls	**Insulin-non-delayed**	**Insulin-delayed**	**LPS**
Nosed	20.5 ± 1.4	20.6 ± 3.4	24.3 ± 3.1	19.8 ± 2.5
Tail Fanning	22.9 ± 5.7	6.4 ± 3.0 [c]	17.4 ± 4.0	17.4 ± 6.3
Nudged	12.6 ± 1.3	17.0 ± 3.7	20.3 ± 4.7	20.5 ± 4.2
Mounted	7.7 ± 0.9	10.0 ± 2.1	12.6 ± 2.6 [b]	3.4 ± 1.3 [a]

[a] Within a row P<0.05 compared to the control and insulin groups.

[b] Within a row P<0.06 compared to controls and P<0.005 compared to LPS groups.

[c] No statistical difference detected du

Chapter 3

Chapter 3

Kisspeptin, c-Fos and CRFR type 2 co-expression in the preoptic area and hypothalamus at different times during the follicular phase of intact ewes and alteration after insulin or endotoxin.

Keywords: c-Fos, kisspeptin, CRFR type 2, stress, oestrous behaviour, LH surge, insulin, LPS, ewes.

Abstract

The hypothalamic neuropeptide kisspeptin is essential for GnRH/LH surge release and, therefore, ovulation. The aim of the present study was to investigate the activational pattern of kisspeptin cells during the follicular phase of intact ewes. Furthermore, we tested the hypothesis that inhibition of kisspeptin activation is a major contributing factor to LH surge disruption during stress and this may involve CRFR type 2. Follicular phases of intact ewes were synchronised with progesterone vaginal pessaries. Control animals were killed at 0h, 16h, 31h and 40h (n=4-6 per group) after progesterone withdrawal (time zero). At 28 h, groups of animals received insulin (4 iu/kg) or endotoxin (LPS; 100 ng/kg) and were subsequently killed at 31h (insulin; n=5 and LPS; n=5) or 40h (LPS; n=5). Hypothalamic sections were immunostained for kisspeptin and a marker of neuronal transcriptional activation, c-Fos. LH surges only occurred in 40 h control ewes: they had a marked increase in the percentage of kisspeptin cells co-expressing c-Fos in the arcuate nucleus (ARC; from 13 to 68%; $P<0.05$) and medial preoptic area (mPOA; from 22 to 47%; $P<0.05$) compared to animals sacrificed at all other stages. However, 12h after LPS treatment (i.e., at 40h), the percentage of kisspeptin cells co-expressing c-Fos was lower in the ARC and mPOA (17% and 10%, respectively; $P<0.05$ for both) whereas there was an increase 3h after insulin (i.e., at 31h; 51%; $P<0.05$) in the ARC but no change in the mPOA, compared to controls killed at the same time. Furthermore, dual-label immunohistochemistry for kisspeptin and CRFR type 2 revealed that 21% kisspeptin cells co-express CRFR type 2 in the lower part of the ARC and the median

eminence (ME) of control animals and this percentage increased to 52 and 58%, 3h and 12h, respectively, after LPS, but not insulin treatment (P<0.05). These results indicate that the LH surge is accompanied by intense transcriptional activation in ARC and mPOA kisspeptin cells. However, insulin or LPS administration in the late follicular phase, disturb this pattern by stimulating or inhibiting kisspeptin cell activation, respectively. CRFR type 2 is associated with inhibition of kisspeptin cells after LPS but not insulin.

Introduction

Gonadotrophin-releasing hormone (GnRH) neurones constitute the final common pathway of a complex neuronal network responding to the circulating steroid hormone milieu to control ovulation and sexual behaviour (Karsch *et al.,* 1997, Caraty *et al.,* 2002). In the ewe, both progesterone and oestradiol exert negative feedback to suppress GnRH release throughout most of the oestrous cycle (Moenter *et al.* 1991, Goodman, 1996). In the late follicular phase, increasing concentrations of oestradiol, secreted by the dominant follicle(s), switch to positive feedback mode and trigger the onsets of the GnRH/LH (luteinising hormone) surges as well as sexual behaviour (Fabre-Nys and Martin 1991). However, steroid hormone signals do not impinge directly on GnRH cells as these cells do not possess progesterone receptors (PR) or oestradiol receptors subtype α (ERα; Shivers *et al*., 1983, Herbison and Theodosis, 1992, Skinner *et al*., 2001). Recent evidence has revealed that kisspeptin is high in the functional 'hierarchy' that mediates steroidal influence on GnRH neurones in a large number of species (Smith, 2008, Roseweir and Millar, 2009, Lehman *et al.* 2010a, Lehman *et al.* 2010b). In the ewe, kisspeptin has been implicated in mediating oestradiol negative and positive feedback (Estrada *et al.* 2006, Smith *et al.* 2007, Smith *et al*. 2009, Smith *et al*. 2011) as well as orchestrating GnRH/LH pulsatility (Lehman *et al.* 2010a) and surge secretion (Smith *et al*. 2009, Smith, 2009, Caraty *et al.* 2010), although to date kisspeptin has not been associated with the control of oestrus behaviour (Kauffman *et al.* 2007). There is controversy concerning which of the two main populations of kisspeptin cells (in the ARC or the mPOA) are involved in oestradiol positive and negative feedback, or whether there are distinct populations within these nuclei that fulfill each of these roles (Estrada

et al. 2006, Smith *et al.* 2009, Caraty *et al.* 2010). Nonetheless, it is widely accepted that kisspeptin is essential for the GnRH/LH surge, and therefore ovulation, in many species including the ewe (Clarkson *et al.* 2008, Oakley *et al.* 2009, Smith *et al.* 2009, Clarkson and Herbison 2009, Lehman *et al,* 2010a, Caraty *et al.* 2010).

Our recent data showed that sudden activation of the hypothalamic-pituitary-adrenal axis in the late follicular phase by administration of lipopolysaccharide (LPS) lowered plasma oestradiol concentrations and delayed the onsets of pre-copulatory behaviours, oestrus and the LH surge of free-running intact ewes, whereas insulin-induced hypoglycaemia had the same effect in only 50% of animals (Fergani *et al.* 2011). However, the precise mechanisms involved in this disruption have not yet been elucidated. Given the crucial role of kisspeptin in regulating GnRH secretion, we speculate that inhibition of kisspeptin activation is involved in stress-induced suppression of reproductive efficiency. Indeed, a few studies report down-regulation of the hypothalamic kisspeptin system in rats and male rhesus monkeys after metabolic or immune/inflammatory stressors, such as negative energy balance (Castellano *et al.* 2010), short term fasting (Wahab *et al*. 2010) or administration of LPS (Iwasa *et al.* 2008, Kinsey-Jones *et al.* 2009). Furthermore, all these stressors were accompanied by suppression of the hypothalamic-pituitary- gonad axis.

Studies assessing changes in messenger RNA (mRNA), or c-Fos induction as a marker of neuronal activation, suggest that a common pathway for the action of stressors is to stimulate the cellular activity of the paraventricular nucleus (PVN), especially neurones secreting corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) that project to the median eminence (ME; Caraty *et al.* 1990, Battaglia *et al.* 1998, Dobson *et al.* 2000). 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 interneurones (Li *et al.* 2005). CRF administration i.c.v. profoundly decreased kisspeptin and Kiss1 receptor mRNA levels in both the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of rats (Kinsey-Jones *et al.* 2009). In this species, kisspeptin (Smith *et al.* 2006) and CRF receptor (CRFR) type 1 and 2 distributions (Chalmers *et al.* 1995, van Pett *et al.* 2000) overlap in the AVPV and ARC and, therefore, an interaction between the two systems seems likely. Li *et al.* (2006) report that insulin-

induced hypoglycaemia or LPS involve the activation of type 2 but not type 1 CRFR, to mediate inhibitory actions. However, the importance of CRF has not been confirmed in the ewe (Caraty *et al.* 1997, Battaglia *et al.* 1998). Whether kisspeptin neurones express CRFR is not known, and remains to be investigated in any species.

Based on these observations, in the present study we examined brain tissue of intact ewes sacrificed at various times during the follicular phase with or without the administration of insulin or LPS. We used c-Fos (a marker for neuronal transcriptional activation; Hoffman *et al.,* 1993) to locate the brain areas that may exhibit transient activational events before or after the onset of pre-copulatory behaviours and oestrus, during the LH surge or after stress. We were particularly interested to investigate any possible differences in insulin treated animals that could explain our previous results with split responses, after the application of this stressor (Fergani *et al.,* 2011). Furthermore, we aimed to map the pattern of kisspeptin activation (by measuring co-expression with c-Fos) in the ARC and mPOA, at various times during the follicular phase, and test the hypothesis that inhibition of kisspeptin activation is a major contributing factor to the LH surge disruption after the application of LPS or insulin. Finally, we examined the presence of CRFR type 2 in kisspeptin cells to establish whether upregulation of this particular receptor is associated with failure of kisspeptin cell activation.

Materials and Methods

Animals, study design and blood sampling procedure

The study was performed in the mid-breeding season (October/November) on 36 mature intact Lleyn crossbred ewes, weighing between 50 and 80 kg. From two weeks prior to the study, the ewes were penned indoors (space 15 x 7 metres) with 3 teaser rams. All animals were fed daily a constant diet of ad libitum hay and had free access to water. Frequent handling, for at least a week ensured that procedural stressors did not interfere with the main part of the study by acclimatizing the animals to human manipulations and a simulated blood sampling process. All procedures were conducted in accordance with requirements of the UK Animal (Scientific Procedures) Act, 1986, and approved by the University of Liverpool Animal Welfare Committee.

Ovarian follicular phases were synchronised by the insertion of 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, to ensure corpus luteum regression. The time of progesterone withdrawal (i.e. the 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 seven groups. One group of animals were 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 of saline vehicle or insulin (neutral zinc bovine insulin, Hypurin Neutral, CP Pharmaceuticals, Wrexham, UK; i.v. dose of 4 iu/kg body weight) or endotoxin (Lipopolysaccharides from Escherichia coli 055:B5, LPS, Sigma-Aldrich, UK; i.v. dose of 100 ng/kg body weight). Three groups were killed at 31h (31h control, n=6; 31h insulin, n=5 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 the insertion of a silastic catheter into the jugular vein of each ewe under local anesthesia before progesterone withdrawal. Patency was maintained with heparinised saline (Multihep, 100 iu/ml, Leo Laboratories, Princes Risborough, UK) administered after each blood withdrawal. Blood samples (5 ml at time 0h, 16h, 24h and subsequently at 2h intervals till 40h) were collected from all ewes into heparinised tubes and centrifuged immediately at 1000 *g* for 20 min at 4^0C. Plasma was stored at -20^0C until analysis. Samples, in duplicate, were analysed by second antibody Enzyme-Linked Immunosorbent Assays (ELISA) for LH, and by single antibody ELISA for 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 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 (Saifullizam *et al.,* 2010). 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. In general, 100 µl aliquots of plasma were assayed initially, and samples that were greater than the maximum standard were re-assayed after a ten-fold or fifteen-fold dilution.

Visual observation of oestrous behaviour

Ewe and ram oestrus behaviour was monitored by two trained observers for a 30-minute observation period prior to each blood sample collection. Paint spray was used to place a large identification symbol on the back and sides of each ewe and ram. The observers were elevated 1.5m above the pen so identification of symbols and all activities could be seen without disturbing the animals. At each observation period, quantitative and qualitative data were recorded for each ewe and ram individually. Once a minute throughout each observation period, it was noted if a ewe was within one metre of a ram [behavioural scan sampling; Martin and Bateson (1986)]. In addition, the following behavioural signs of oestrus were counted throughout each 30 min observation period: ram nosing the perineal region of a ewe; ewe being nudged by a ram without the ewe moving away; and, mounting of the ewe by a ram without the ewe moving away. Due to the 2-hourly observation regime, the beginning of a period was respectively defined as the first (minus 1.0h) 30-min observation period the animal exhibited a particular behavioural sign.

Tissue collection

With minimal disturbance of the animals, euthanasia was carried out with 20 ml of 20% w/v sodium pentobarbitone (Pentobarbital, Loveridge, Southampton, UK), containing 25,000IU heparin. Each head was retrieved immediately, jugular veins occluded and perfused bilaterally via the carotid arteries using a peristaltic pump (Gilson Minipulus-3, Villiers, France). The solutions used for perfusion were: 2 litres 0.1M phosphate buffer (PB; pH7.4) containing 25,000IU per litre of heparin and 1% sodium nitrate; then 2 litres of Zamboni fixative (4% paraformaldehyde powder 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 of wash–out solution (0.1M PB, 40% sucrose and 0.1% sodium azide) was pumped through. The brain was retrieved immediately afterwards and 17mm hypothalamic blocks (extending from the optic chiasma to the mammilary bodies) were obtained. These were stored in the wash-out solution at 4^0C for a week. The blocks were frozen using isopentane (2-Methylbutane, Chromasolv®, for HPLC, Sigma-Aldrich, UK) and liquid nitrogen as described by Rosene *et al.* (1986) and stored at -80^0C. Frozen coronal sections (40 μm) were cut using a freezing microtome (Microm HM400R, Walldorf, Germany). Free-floating sections were stored in 96-well microtest plates (catalogue number 82.1582, Sarstedt, UK) with each section placed in an individual well filled with cryoprotectant solution (Watson *et al.* 1986) and stored at -20^0C until processed for immunohistochemistry.

c-Fos and kisspeptin dual-label immunohistochemistry

A series of every 15th section through the hypothalamus and preoptic area from each animal was processed for c-Fos and kisspeptin staining using a dual-immunoperoxidase protocol in which nuclear c-Fos was detected first with nickel sulfate-enhanced diaminobenzidine as chromogen (ni-DAB; black reaction product), followed by detection of cytoplasmic kisspeptin using unenhanced diaminobenzidine (DAB; brown reaction product). 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 a 72h incubation in rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA) at a dilution of 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 visualised by 5min incubation in ni-DAB (SK-4100, Vector Laboratories Ltd, UK). A second immunohistochemical procedure was then performed, as described above, to stain the second primary antibody: rabbit anti-kisspeptin serum (1:25,000; lot 564; gift from Prof. Alain Caraty, Nouzilly, France), incubated for 72h at 4^0C and then visualised using DAB. Finally, sections were washed in distilled water, mounted on Chrome Alum Gelatine coated slides and left to air-dry for 3 days. Slides were dehydrated in a series of solutions (5min in each) containing 73%, 85%, 96% and two changes in 100% alcohol followed by xylene and a coverslip was applied using DPX. Negative control sections were performed routinely by omitting primary antibody(s) which was substituted with antibody diluting solution alone. This resulted in complete loss of staining.

CRFR type 2 and kisspeptin dual-label immunohistochemistry

In the case of dual-labelling for CRFR type 2 (1:4000; ab12964; Abcam UK; Lakshmanan *et al.,* 2007) and kisspeptin, three sections, about 240 microns apart, containing the ARC and ME together were chosen and a dual-immunoperoxidase staining protocol performed as described above. In this protocol a different type of secondary biotinylated antibody was used (R.T.U.; PK-7200, Vector Labs). The CRFR type 2 immunohistochemistry was performed first and the visualization was performed with ni-DAB to produce a black product. A second immunohistochemical procedure followed to stain for kisspeptin using DAB alone. The exclusion of the primary or secondary antibody resulted in a complete absence of staining.

Data analysis

Hormone, behaviour and immunohostochemistry data were analysed 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 was considered to begin (surge onset) when the first value increased more than ten times the minimum detectable quantity (i.e. >10 ng/ml). Cortisol, progesterone and oestradiol 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, progesterone concentrations at 28h (i.e. just prior to treatment) were compared 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.,* 1969): 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 and kisspeptin data were derived from 3 photographs that included the lower part of the ARC and the ME. Data from these two areas were combined because of the small number of cells but also due to the confined location of the receptors; i.e., on the 'border' either side of the ARC and ME boundary (i.e., there was difficulty in distinguishing whether some receptors belonged to the ARC or to the ME). Sections were evaluated unilaterally and each photograph was taken from a random field within each area. All photographs were imported in to Image J version 1.42q, and counts were 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 values from ewes in each group.

During data analysis, it became clear that there was a split response in the insulin group regarding the c-Fos activation of the PVN. Therefore this group was separated into two subgroups referred to hereafter as insulin-responders or insulin-non-responders depending on c-Fos activation. As this division reduced the group size to n=2/group, statistical analysis was not undertaken, but the data are presented for information.

When responses were not different between sub-groups, data were combined and analysed with n=4.

Results

Three animals exhibited signs of oestrus and were mounted by a ram within 28 h after progesterone withdrawal (i.e., before the predetermined time of treatment; one from each of the 31h INS, 31h LPS and 40h LPS groups). The data from these three 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.

Luteinising hormone (LH) and behavioural profiles.

The onset of pre-copulatory behaviours, oestrus and the LH surge of individual ewes with respect to progesterone withdrawal (PW) are shown in Table 1. None of the treated animals began an LH surge during the 40h of study. However, 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). The data were analysed in two ways: the first consisted of control ewe data grouped according to time after PW, then by sexual behavioural status and whether an LH surge had occured; i.e., grouped into those killed at 0h and 16h after PW, those killed at 31h after PW but before the onset of sexual behaviour (Before behaviour, n=3), those killed at 31h or 40h after PW, after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). This grouping allowed for a detailed comparison of neuropeptide profiles in control animals at different stages of the follicular phase in our intact-ewe model. 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: transcriptional activation in the hypothalamus and preoptic area at different stages during the follicular phase, in animals grouped by behaviour.

VMN

The number of c-Fos positive cells in the VMN was three 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 2A).

ARC

The number of c-Fos positive cells in the ARC was 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 2B).

mPOA

The number of c-Fos positive cells in the mPOA was three times greater in the 'Surge' group compared to all other stages in the follicular phase of control ewes ($P<0.001$ for all comparisons; Fig 2C).

ME, dBb, BNST

The number of c-Fos positive cells in the ME and dBb was not different at any stage in the follicular phase of control ewes (Fig 2D and 2E). However, the number of c-Fos positive cells in the BNST was three times greater in the 'Before behaviour' group compared to all other stages in control ewes ($P<0.001$ for all comparisons; Fig 2F).

Control ewes: kisspeptin and c-Fos co-expression in the hypothalamus and preoptic area at different stages during the follicular phase, in animals grouped by behaviour.

Distribution of kisspeptin immunoreactive cell bodies and fibres

Examination of serial sections revealed the presence of kisspeptin immunoreactive cell bodies and fibres in the following anatomical structures as defined by Welento *et al.* (1969) and similar to those in previous reports (Franceschini *et al.* 2006, Lehman *et al.* 2010a): the rostral, but primarily the medial and caudal ARC, extending up to the pre-mammilary recess; the mPOA at the level of the organum vasculosum of the lamina terminalis; and the PVN (~ 30 cells per section and numerous fibres). A small number of scattered kisspeptin positive cells and fibres were also detected alongside the walls of the third ventricle, 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 internal zone of the ME, along with a dense fibre network, but this network was less dense in the external zone. The number of kisspeptin positive cell bodies differed among animals but not groups (full data not shown). No kisspeptin cells were observed in the dBb or the BNST.

ARC

There was a marked increase in the percentage of kisspeptin cells that co-expressed c-Fos in the 'Surge' group compared to all other stages in the follicular phase ($P<0.01$; Fig 3A).

mPOA

The percentage of kisspeptin cells that co-expressed c-Fos, gradually increased from 16h through 'Before behaviour' and 'After behaviour' to the 'Surge' group ($P<0.05$, for 'Surge' compared to all other stages in the follicular phase; Fig 3C).

Control, insulin or LPS ewes: transcriptional activation in the hypothalamus and preoptic area at different times during the follicular phase, in animals grouped by hours after PW.

PVN

The number of c-Fos positive cells in the PVN was not different between control animal groups (Fig. 4). 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 4) compared to control groups. Interestingly, the same was not observed after insulin treatment. During data analysis, it became clear that this was due to a split response; two out of four insulin-treated animals had an increase in PVN c-Fos-positive cells (12.9 ± 8.4% versus 81.7 ± 5.7%, compared to controls; Fig 4A, 4B) but not the remaining two ewes (12.9 ± 8.4% versus 9.7 ± 4.0%, compared to controls; Fig 4A, 4C). Therefore, the insulin treated animals were separated into two sub-groups, referred to hereafter as insulin-responders or insulin-non-responders, respectively.

VMN

At 31h after PW (i.e., 3h after insulin or LPS administration), there was a marked increase in VMN c-Fos positive cells in the LPS group ($P<0.01$) and the insulin-responder sub-group (Fig 5A). The effect of LPS was still evident when compared to the control

groups at 40h after PW, (i.e., 12h after the initial application of saline or LPS; *P*<0.05; Fig 5A).

ARC

At 31h after PW (i.e., 3h after insulin or LPS administration), there was a marked increase in the number of ARC c-Fos positive cells in LPS (*P*<0.01; Fig 5B) compared to controls. An increase was also observed in both insulin sub-groups (from 59.9 ± 16.9 to 215.8 ± 49.8 cells; combined insulin-responders and insulin-non-responders; *P*<0.01; Fig 5B) compared to controls. At 40h after PW (i.e., 12h after the initial application of saline or LPS), when the majority of control animals were undergoing an LH surge, control and LPS group data were not different (Fig 5B).

mPOA

At 31h after PW (i.e., 3h after insulin or LPS administration), the number of c-Fos positive cells in the mPOA increased in the LPS group (*P*<0.03; Fig 5C), but not in either insulin subgroup, compared to controls. At 40h after PW (i.e., 12h after the initial application of saline or LPS), when the majority of control animals were undergoing an LH surge, control and LPS group data were not different (Fig 5C).

ME, dBb, BNST

The numbers of c-Fos positive cells in the ME and BNST were not altered after the application of insulin or LPS (Fig 5D and 5F). However, at 31h and 40h after PW (i.e., 3h and 12h after LPS administration) LPS treatment groups had markedly increased numbers of c-Fos positive cells in the dBb compared to controls (*P*<0.05; Fig 5E); whereas, insulin had no effect (Fig 5E).

Control, insulin or LPS ewes: kisspeptin and c-Fos co-expression in the hypothalamus and preoptic area at different times during the follicular phase, in animals grouped by hours after PW.

PVN

There was no difference between groups, in the percentage of kisspeptin cells that co-expressed c-Fos in the PVN (16.9 ± 4.3 %; full data not shown).

ARC

At 31h after PW (i.e. 3h after insulin or LPS administration), the percentage of kisspeptin cells that co-expressed c-Fos increased in both insulin sub-groups compared to controls (from 10.7 ± 1.7 to 51.0 ± 1.0%; combined insulin-responders and insulin-non-responders; $P<0.05$; Fig 3B), whereas the LPS group was not different to controls (Fig 3B). However, at 40h after PW (i.e., 12h after LPS administration) the percentage of kisspeptin cells that co-expressed c-Fos was markedly lower in LPS treated animals compared to controls ($P<0.01$; Fig 3B). Photomicrographs of sections of the ARC in control and LPS treated animals are shown in Fig 6.

mPOA

At 31h after PW (i.e., 3h after insulin or LPS administration), the percentage of kisspeptin cells that co-expressed c-Fos was not different between control and treated groups (Fig 3D). However, at 40h after PW (i.e., 12h after LPS administration), the percentage of kisspeptin cells that co-expressed c-Fos was markedly lower in LPS treated animals compared to controls ($P<0.01$; Fig 3D).

CRFR type 2 and kisspeptin

Distribution of cells containing CRFR type 2 in the ARC + ME

Examination of sections containing ARC and ME structures revealed the presence of 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, 7E, 7F).

CRFR type 2 immunoreactivity

There was no difference in the number of CRFR type 2 cells between control and insulin treated animals (Fig 7A). However, LPS treatment increased CRFR type 2 immunoreactivity in the lower part of the ARC + ME ($P<0.001$; Fig 7A). This was evident at 31h and 40h after PW (i.e. 3h and 12h after LPS administration).

Kisspeptin and CRFR type 2 co-expression.

In control and insulin-treated animals, there was no difference in the percentage of kisspeptin cells that co-expressed CRFR type 2 (average throughout: 21.3 ± 2.6%; Fig 7B). However, at 31h and 40h after PW (i.e., 3h and 12h after LPS administration), the percentage of dual–labelled cells doubled in the LPS groups (P<0.05; Fig 7B, 7G). There was no difference between groups in the percentage of CRFR type 2-positive cells that co-localised kisspeptin (average throughout: 10.6 ± 1.9%; Fig 7C).

Hormone data

Cortisol

In all control animals, mean plasma cortisol concentrations remained low throughout (10.5 ± 0.7 ng/ml; Fig. 8). Before the application of insulin or LPS (i.e., at 24 and 28h after PW), cortisol concentrations did not differ from values in controls (Fig. 8). At 30h, cortisol concentrations in both insulin-treated groups were elevated compared to controls (from 9.5 ± 0.7 to 70.4 ±5.8 ng/ml; combined insulin-responders and insulin-non-responders; P<0.001; Fig. 8). At 30h, both groups of LPS animals, had higher values compared to control and insulin-treated groups (P<0.001; Fig. 8). In the 40h LPS group, ewes had increased concentrations compared to the controls at 32, 36 and 40h (for each P<0.05; Fig. 8). Mean maximum cortisol concentrations (157 ± 19.8 ng/ml) for the LPS groups were observed 2h after treatment (Fig. 8).

Progesterone

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. Control and insulin sub-groups had similar concentrations of progesterone before and after treatment (Fig.9). Progesterone concentrations were different in 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. 9). Progesterone concentrations after PW in control animals are also shown in Fig. 10.

Oestradiol

In control animals, plasma oestradiol concentrations continued to increase from 28h after PW to maximum values between 32h and 36h after PW (Fig 10A). However, after treatment with insulin, oestradiol concentrations were lower 2h after the first injection compared to controls killed at the same time (from 9.5 ± 0.8 to 4.1 ± 0.4 pg/ml; combined insulin-responders and insulin-non-responders; $P<0.05$; Fig. 10B). In contrast, in LPS ewes the decrease occurred more slowly: values tended to be lower than controls 8h after treatment (i.e., at 36h after progesterone withdrawal; $P<0.07$; Fig.10A) and thereafter continued to decrease significantly until ewes were killed at 40h ($P<0.02$; Fig 10A).

Discussion

The present results demonstrate that during the LH surge of intact ewes (and not at other stages in the follicular phase) there is an intense transcriptional activation of kisspeptin cells located in the ARC and mPOA. This suggests that cells in these areas contribute to oestradiol positive feedback to stimulate the GnRH/LH surge in this species. However, LPS treatment in the late follicular phase inhibited kisspeptin activation in the ARC and mPOA, whereas insulin stimulated kisspeptin activation in the ARC but had no effect in the mPOA. Dual-label immunohistochemistry revealed that 21% of kisspeptin cells co-express CRFR type 2 in control and insulin-treated ewes but this doubled in LPS treated animals. Thus, CRFR type 2 is associated with inhibition of kisspeptin transcriptional activation and the disruption of the LH surge after LPS but not after insulin.

Controls: kisspeptin and c-Fos co-expression in the hypothalamus and POA at different stages of the follicular phase.

At all times during the follicular phase c-Fos positive cells were observed in all the brain areas examined with a marked increase in the VMN, ARC and mPOA during the LH surge. Interestingly, at 31h after PW (i.e., the time when oestradiol was reaching maximum concentrations) activation was low in the VMN, ARC and mPOA. As the GnRH surge generating mechanism consists of three phases (activation, transmission and surge secretion; Evans *et al.,* 1997, Harris *et al.,* 1998, Harris *et al.,* 1999), our results suggest

that these areas are involved in the surge secretion phase. These findings concur the work of Richter *et al.,* (2005) who examined ovariectomised (OVX) animals during the activation phase of the surge generating mechanism (4h after oestradiol administration) as well as during the surge onset (20-25h after oestradiol). They also report higher transcriptional activation of the ARC and mPOA in the latter stage but not the former.

Oestradiol implants in the mediobasal hypothalamus (MBH; the vicinity of the ARC and VMN) initiated pre-copulatory behaviours and oestrus (Blache *et al.,* 1991). We did not detect an increase in MBH (ARC or VMN) activation in animals before or after the onset of sexual behaviour and before the LH surge. This does not exclude involvement of these areas in sexual behaviour. It may be that the phenotype of cells changes to stimulate sexual behaviour, even though the overall number of c-Fos activated cells remains the same. A similar conclusion might be drawn regarding the lack of variation during the follicular phase in the number of c-Fos positive cells in the ME or dBb. In contrast, an increase in BNST activation was observed in animals just before the expected onset of pre-copulatory behaviours/oestrus suggesting that this area may be an intermediary between the MBH and GnRH neurones in the mPOA for the control of oestrous behaviour (Caraty *et al.,* 2002) and/or the GnRH/LH surge (Pompolo *et al.,* 2001, Pereira et al., 2010). Indeed, the BNST is an ERα-rich area (Goubillion *et al.,* 1999) that receives projections from the MBH and also sends projections to the mPOA where most GnRH cell bodies are located (Pompolo *et al.,* 2001, Pereira *et al*., 2010). However, it must be noted that the animals in our 'Surge' group were also exhibiting sexual behaviour and, therefore, the transcriptional increase observed in the VMN and ARC could, in part, be involved with changes in behaviour as well.

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 or negative oestradiol feedback. In the present study, the LH surge of intact ewes was accompanied by an intense transcriptional activation of kisspeptin neurones located in both these areas. Regarding the ARC, all regions (rostral, middle and caudal) contributed equally to the increase. This, together with the observation that oestradiol acts in the MBH to induce the LH surge in the ewe (Blache *et al.,* 1991; Caraty *et al.,* 1998) suggests that these ARC cells are involved in oestradiol positive feedback. However, as the

oestradiol signal initiating the GnRH surge begins well in advance of the surge itself (Evans *et al.,* 1997), we cannot conclude that ARC kisspeptin cells are solely responsible for the feedback effects of oestradiol, but are most likely only involved in the surge secretion mechanism. Indeed, in the present study, plasma oestradiol concentrations in control animals were elevated at 28h, reached a maximum at 32h-36h after PW and then decreased just as the LH surge occurred, but we did not observe a striking change in ARC kisspeptin neurone activity around the time of oestradiol maximum concentrations. However, we cannot rule out the possibility that activation in ARC kisspeptin neurones 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 of transmit the positive oestradiol signal to GnRH neurones. Indeed, the LH response to exogenous kisspeptin increases during the cycle and is highest in the pre-ovulatory phase in rats and women (Roa *et al.,* 2006, Dhillo *et al*., 2007). Recent reports indicate that ARC kisspeptin neurones co-express c-Fos in OVX ewes 1–2h after administration of a short oestradiol signal, i.e., 12–18h before the onset of the LH surge (Smith *et al.,* 2009). This raises the possibility that ARC kisspeptin neurones are activated in response to the initial oestradiol increase and are involved in the early stages of the surge induction process. However, in the same study, kisspeptin mRNA increased only immediately prior to the LH surge of non-steroid-treated intact ewes. This incompatibility in results may be due to the use of ovariectomised or intact animals with high supplementary oestradiol doses that could produce different c-Fos activation patterns than are normally seen in intact animals with physiological oestradiol concentrations. Almost all kisspeptin cells in the ARC, but not elsewhere, co-express dynorphin and neurokinin B (Goodman *et al.,* 2007). Therefore, activation of kisspeptin cells may reflect increased activity of any of the three neurotransmitters, dynorphin, neurokinin B and/or kisspeptin in this region.

Turning to the mPOA, we observed a gradual increase in kisspeptin activation during the follicular phase with maximum activation during the surge. Interestingly, the proportion of activated cells was in the same range as the proportion of POA kisspeptin cells that express ERα in this species (Franceschini *et al*., 2006). Two recent studies also report an increase in c-Fos activity of the mPOA kisspeptin cells at the time of the preovulatory LH

surge (Hoffman *et al*., 2010) and an increase in kisspeptin mRNA in the late follicular phase (Smith *et al.,* 2009). However, as mentioned above, in the ewe, oestradiol acts in the MBH, not the POA, to induce the LH surge (Blache *et al.,* 1991; Caraty *et al*., 1998) so we speculate that mPOA kisspeptin neurones are activated secondarily (possibly via the ARC) during oestradiol positive feedback. Indeed, Lehman *et al.* (2010a) report the existence of projections from ARC kisspeptin neurones to POA kisspeptin neurones, indicating that activation of one population could influence another. The recent demonstration that both ARC and mPOA kisspeptin cell populations in sheep are sexually dimorphic is consistent with the view that the two populations could be involved in the GnRH surge inducing process (Cheng *et al.,* 2010).

Kisspeptin has also been implicated in oestradiol negative feedback. Ovariectomy increases kisspeptin peptide and kisspeptin mRNA expression in the ARC of ewes (Pompolo *et al.,* 2006, Smith *et al.,* 2008) and rats (Smith *et al.,* 2006) while oestradiol replacement diminishes this effect. 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 oestradiol inhibits the expression of kisspeptin, leading to reduced GnRH secretion, consistent with negative feedback regulation.

Insulin or LPS: kisspeptin and c-Fos co-expression in the hypothalamus and POA in ewes after treatment in the late follicular phase.

Expression of c-Fos in the ovine central nervous system is a useful marker for the identification of brain regions activated by stressors (Vellucci and Parrott, 1994). In the present study, LPS administration in the late follicular phase lowered plasma oestradiol concentrations and activated specific brain areas at different times. Interestingly, an increase in activation was observed in the ARC, VMN, mPOA and dBb as soon as 3h after treatment, whereas plasma oestradiol concentrations decreased 8h after the administration of LPS. Thus, there could be at least two mechanisms activated during LPS inhibition of the ovarian cycle; one involving disruption of GnRH/LH pulses and, therefore, reducing oestradiol secretion; and the other, preventing the ability of the surge-generating mechanism to respond to the preovulatory oestradiol increase (Battaglia *et al.,* 1999, Karsch and Battaglia, 2002). Furthermore, LPS markedly

decreased the proportion of activated kisspeptin cells three- to four-fold in the ARC and mPOA, and this was evident 12h after treatment. Therefore, we cannot be sure which of the two mechanisms disrupted kisspeptin cell activation.

Cortisol and progesterone suppress pulsatile GnRH/LH secretion (Karsch *et al.,* 1987, Debus *et al.,* 2002, Oakley *et al.,* 2009) and disrupt the positive feedback effect of oestradiol to trigger an LH surge (Kasa-Vubu *et al.,* 1992, Skinner *et al.,* 1998, Richter *et al.,* 2002, Smith *et al.,* 2003, Richter *et al.,* 2005, Pierce *et al.,* 2009, Wagenmaker *et al.,* 2009a). In the present study, cortisol increased to maximum concentrations 2h after administration of LPS, and may be involved in the GnRH surge mechanism disruption. By contrast, the timing of maximum progesterone values varied considerably between animals, from 2h to 10h after treatment. Thus, we cannot determine which mechanism is disrupted by progesterone. The potential influence of pyrexia remains to be determined: maximum temperatures after the same dose of LPS were reached 4h after treatment (Fergani *et al.,* 2011) indicating that pyrexia, presumably via prostaglandin, may contribute to the attenuation of GnRH pulses. Nonetheless, as the LPS treated animals did not have an LH surge at the same time as controls, we conclude that the inhibition of the kisspeptin system is a major contributing factor in LH surge disruption after an immune/inflammatory challenge in the ewe. Our data agree with findings in rats showing that kisspeptin and kisspeptin receptor mRNA are reduced in the ARC and POA concomitant with gonadotrophin hormone secretion suppression (Iwasa *et al*., 2008, Kinsey-Jones *et al.,* 2009).

Oestradiol concentrations decreased within 2h after insulin treatment, but only after 8h following LPS administration. The reason for this time lag in oestradiol decrease between the two stressors is not understood, however, we have already shown that hypoglycaemia is induced immediately after insulin administration (Fergani *et al.,* 2011) and could be one cause of GnRH/LH surge and pulse inhibition leading to immediate oestradiol suppression (Dobson and Smith, 2000). Interestingly, we observed a split response in the insulin-treated animals with two showing an intense activation in the PVN and VMN (insulin-responders) whereas the other two were not different to controls (insulin-non-responders). A similar divergence was observed in our previous study (Fergani *et al.,* 2011) when 10 out of 20 animals treated with insulin did not have a delay

in sexual behaviour or the LH surge (insulin-non-delayed subgroup). Taking into account the fundamental importance of the PVN in response to stress (Antoni, 1986) and the importance of the VMN in inducing the GnRH surge (Caraty *et al.,* 1998), we speculate that the animals with no activation in the PVN or VMN would have gone on to have an LH surge at a similar time as the controls, whereas the others would have had a delayed surge. Interestingly, cortisol concentrations increased equally in both insulin sub-groups. This was also observed in our earlier study indicating that cortisol is not solely responsible for the LH surge disruption after insulin (Fergani *et al.,* 2011). The reason for this divergence is not known, and it is particularly interesting that even though PVN activation did not occur, plasma cortisol concentrations were elevated. Furthermore, the split response does not involve insulin-resistance (Fergani *et al.,* 2011). In this aspect, Tilbrook and Clarke (2006) discuss the existence of individuals that are more sensitive to the negative feedback of glucocorticoids, resulting in stress hypo-responsiveness. Thus, insulin may be a less severe stressor than LPS, and certain individuals being more stress-resilient, are able to recover from the stress axis activation very quickly.

Transcriptional activation in the ARC also increased in both insulin subgroups probably because the ARC and the VMN play a pivotal role in glucose-sensing and energy balance (Cone *et al.,* 2001, Routh, 2003). Indeed, it has been hypothesised that ARC is central in metabolic regulation and relays peripheral signals to appetite regulating systems in other parts of hypothalamus including the VMN and PVN (Elmquist, 2001). It is likely that even though insulin-induced hypoglycaemia was 'perceived' by the ARC, resulting in activation, the transmission of the signal towards the VMN and the PVN was disturbed.

Nonetheless, the observation that LPS activated more brain areas than insulin may be associated with the intensity of the stressor (Tilbrook *et al.,* 2002), i.e., more inhibitory pathways are activated after LPS treatment and this may be associated with LPS delaying the LH surge for longer than insulin (Fergani *et al.,* 2011). The precise phenotype of all the activated cells remains to be elucidated.

Intriguingly, acute insulin administration in the late follicular phase immediately increased five-fold the number of kisspeptin cells co-expressing c-Fos in the ARC but had no effect on mPOA kisspeptin neurones. These data are consistent with findings in the

rat regarding the mPOA, but differ regarding the ARC: there was only a tendency towards suppression of kisspeptin mRNA levels (Kinsey-Jones *et al.,* 2009). Furthermore, short-term fasting decreases kisspeptin mRNA expression in the whole hypothalamus of rats (Castellano *et al.,* 2010) and monkeys (Wahab *et al.,* 2010). The differences between these studies could be attributed to the level of hypoglycaemia that was recorded after acute insulin and fasting, respectively. For example, in our model there is a 60% decrease in glucose concentrations (Fergani *et al.,* 2011), whereas in fasted monkeys there was only a 20% decrease. To date, it is not known whether kisspeptin neurones express insulin receptors, although the latter are abundant in the ARC (Qi *et al.,* 2008) and could be responsible for the observed effects. Finally, we cannot rule out the possibility of transient stimulation which may have been followed by a decrease in kisspeptin activation after a short period of time.

The percentage of activated kisspeptin cells in the PVN (17%) did not vary during the follicular phase or after the application of stressors. This is not surprising as this area is deprived of ER (Lehman *et al.* 1993, Blache *et al.* 1994, Herbison *et al.* 1993) and so this population of cells is probably not involved in direct control of reproduction. However, it would be interesting to identify possible connections between kisspeptin cells in the ARC and cells in the PVN, as a potential pathway for conveying a stress signal between these two areas.

Kisspeptin and CRFR type 2 co-expression.

Although precise neural inputs to the mPOA, ARC and VMN during stress-induced suppression of kisspeptin remain to be determined, CRF is a prime candidate. In the rat, CRF has a pivotal role in stress-induced suppression of the GnRH pulse generator, while CRF antagonists reverse the response (Cates *et al.,* 2004). A differential role for CRFR type 1 and CRFR type 2 was observed in suppression of pulsatile LH secretion in the rat: insulin-induced hypoglycaemia and LPS stressors involve activation of CRFR type 2 while psychological stress (e.g., restraint) involves both CRFR type 1 and type 2 (Li *et al.,* 2005, Li *et al.,* 2006). However, all three of these stressors, as well as intracerebroventricular (icv) administered CRF, markedly decrease kisspeptin and kisspeptin receptor mRNA levels (Kinsey-Jones *et.al,* 2009). In the sheep, the evidence is less compelling. In OVX

ewes, CRF stimulation and inhibition of GnRH occur simultaneously after LPS administration (Battaglia *et al.,* 1998); in contrast, icv administration of CRF either increases (Naylor *et al.,* 1990, Caraty *et al.,* 1997) or has no effect (Clarke *et al.,* 1990, Caraty *et al.,* 1997) on LH pulse frequency. In addition, CRF antagonist was unable to prevent the inhibitory effect of hypoglycaemic stress on LH pulses in the sheep (Clarke *et al.,* 1990). However, in the present study, there was an abundance of CRFR type 2 in the lower part of the ARC and the ME. In addition, 21% kisspeptin cells express this type of receptor and that doubled 3h and 12h after LPS treatment. This indicates that CRFR type 2 may be involved in down-regulation of kisspeptin transcriptional activation. However, there was a large number of CRFR type 2 that co-localised with cells of unknown phenotype. There are, therefore, two possible pathways for CRF suppression of GnRH, one being the direct association of CRF and GnRH cell terminals in the external zone of the ME (Ghuman *et al.,* 2010) and the other being the regulation of kisspeptin and other cell types in the ARC and ME via CRFR type 2. Indeed, cells that originate from the ARC, including kisspeptin cells, send projections towards GnRH terminals (Jansen *et al.,* 1996, Smith *et al.,* 2011,). Whether CRFR type 1 or 2 co-localise with GnRH neurones in the mPOA in the ewe as in rats (Jasoni *et al.,* 2005) remains to be investigated.

Conclusion

Our data indicate that the LH surge of intact ewes is accompanied by an intense transcriptional activation of kisspeptin neurones located in the ARC and the mPOA. Taking into account that the GnRH surge mechanism consists of three phases (activation, transmission and surge secretion) our results suggest that these kisspeptin cells are involved in the surge secretion phase. The cells that become active before the LH surge, when oestradiol concentrations are initially elevated remain to be phenotyped. By contrast, acute LPS treatment prevented the LH surge from occurring and this was accompanied by a failure of kisspeptin neurone activation. Interestingly, insulin increased activation of kisspeptin neurons in the ARC immediately after treatment indicating stressor specific differences. Furthermore, we found that co-expression of CRFR type 2 in kisspeptin cells increased after LPS but not insulin, indicating that this is one of the pathways leading to kisspeptin inhibition and the disruption of the LH surge.

Acknowledgements

Thanks are due to Nigel Jones and the farm staff for care of the animals; Hilary Purcell, David Jones and Peter Taylor for technical assistance; and Prof A Parlow and NIAMDD, USA for LH standard preparations. We are also grateful to Richard Morris for his guidance in immunohistochemical techniques, Prof. A. Caraty for the kisspeptin antibody and Dr Michael Morris for help with animal behavioural observations.

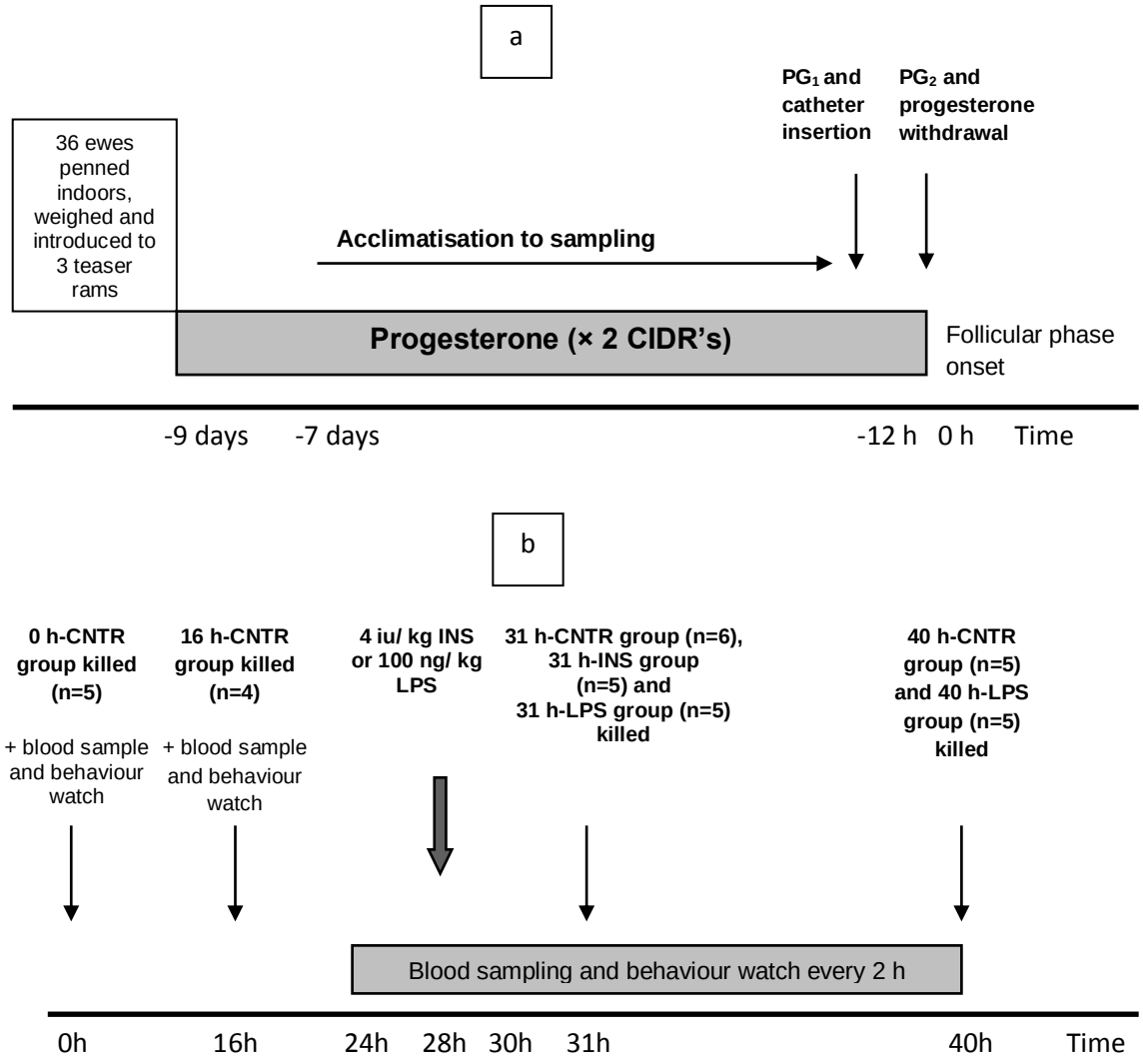

Fig.1 Diagram of the chronological order of events in the experimental protocol (a) before and (b) after the onset of the follicular phase. CNTR = control; INS = insulin; LPS = E. coli lipopolysaccharide

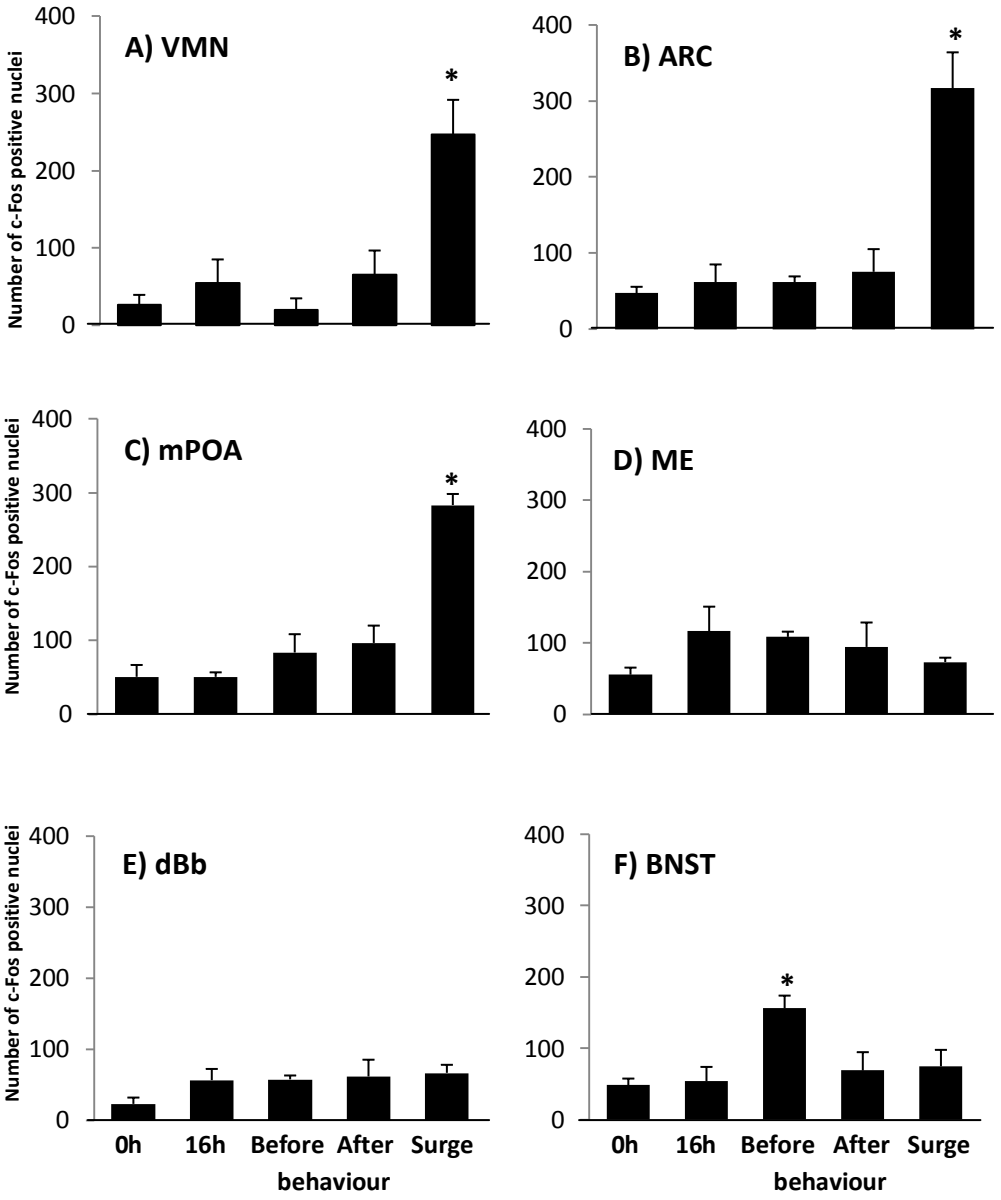

Fig. 2 Mean (±SEM) number of c-Fos positive nuclei in the A) VMN, B) ARC, C) mPOA, D) ME, E) dBb and F) BNST, at different stages in the follicular phase of control ewes. Animals were grouped according to time as well as hormonal and behavioural status; i.e., grouped into those killed at 0h and 16h after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

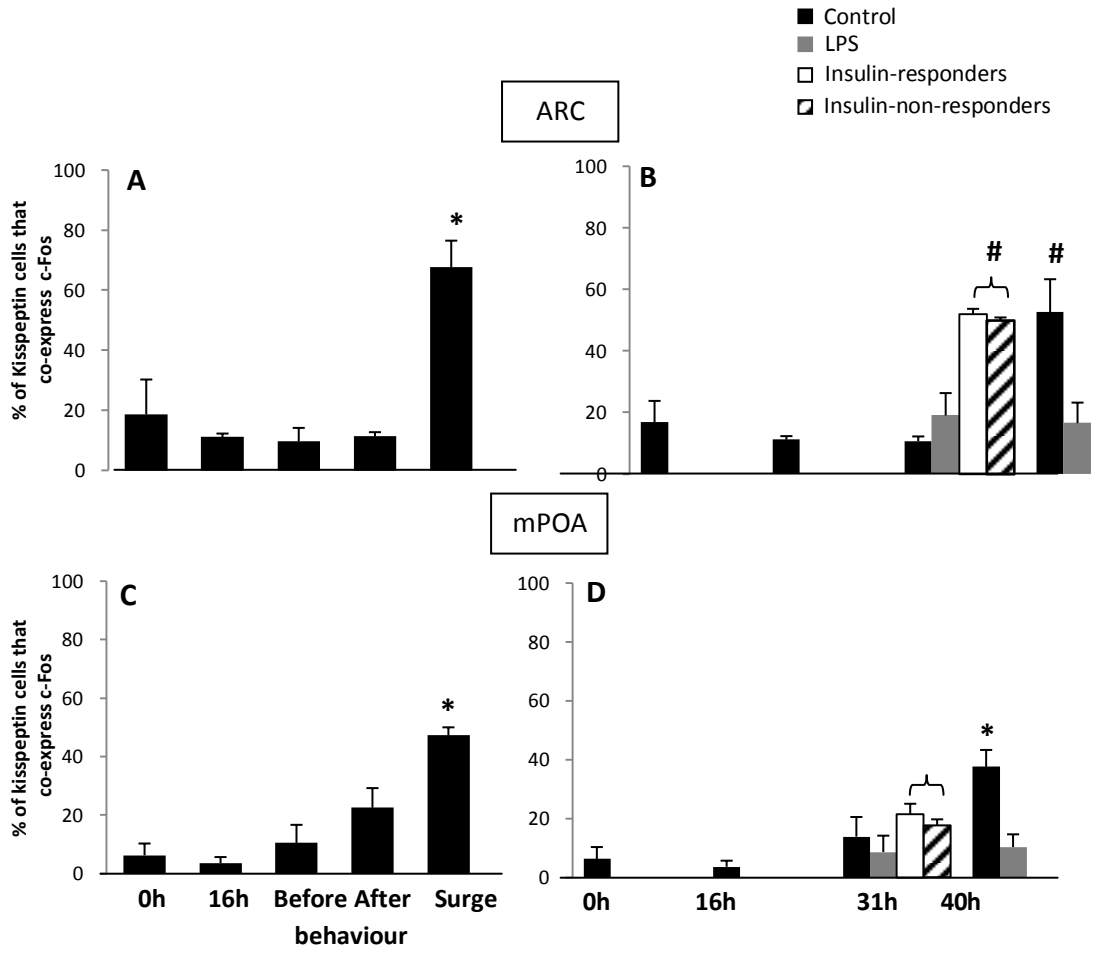

Fig. 3 Mean (±SEM) % number kisspeptin cells that co-express c-Fos in the ARC A, B) and mPOA C, D) during the follicular phase of intact ewes. Animals in A and C are grouped according to time as well as hormonal and sexual behavioural status; i.e. grouped into those killed at 0h and 16h after progesterone withdrawal, those killed at 31h or 40h after PW but before the onset of sexual behaviour (Before behaviour, n=3), those killed at 31h or 40h after progesterone withdrawal, after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). In B and D animals are grouped according to killing 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). There were no split responses observed in the 31h insulin-treated animals i.e., insulin-responders (n=2; white bars) and insulin-non-responders (n=2; hatched bars) and therefore statistical analysis was carried out in both groups combined (n=4). Treatment with insulin or LPS was at 28h after progesterone withdrawal. ***P***<0.05 compared to all other control and treated groups. # *P*<0.05 compared to 0h, 16, 31h control, 31h LPS, and 40h LPS groups.

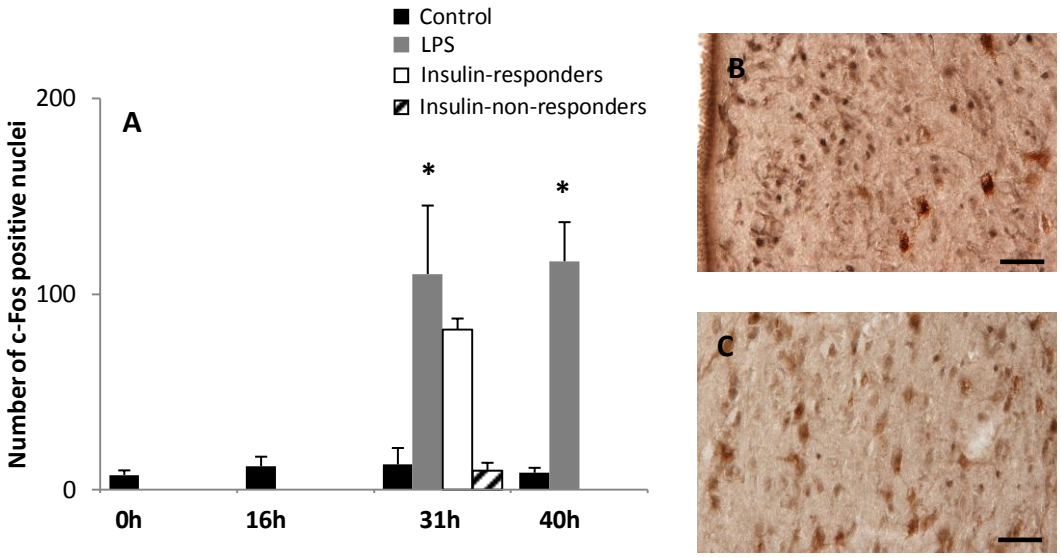

Fig. 4 A) mean (±SEM) number of c-Fos positive nuclei in the PVN of control ewes at 0h (n=5), 16h (n=4), 31h (n=6) and 40h (n=5) in to the follicular phase as well as after LPS (31h LPS and 40h LPS; n=4 for both groups). There was a split response observed in the insulin treated animals: half showed an increase in c-Fos-positive nuclei (insulin responders; n=2) and half did not (insulin-non-responders; n=2), therefore, statistical analysis was not carried out and the data are presented for information. B) and C) photomicrographs of the paraventricular nucleus stained for c-Fos and kisspeptin in insulin responders and insulin non-responders, respectively. *Scale bar* 50 μm. Treatment with insulin or LPS was at 28h after PW. * P<0.05 compared to control groups.

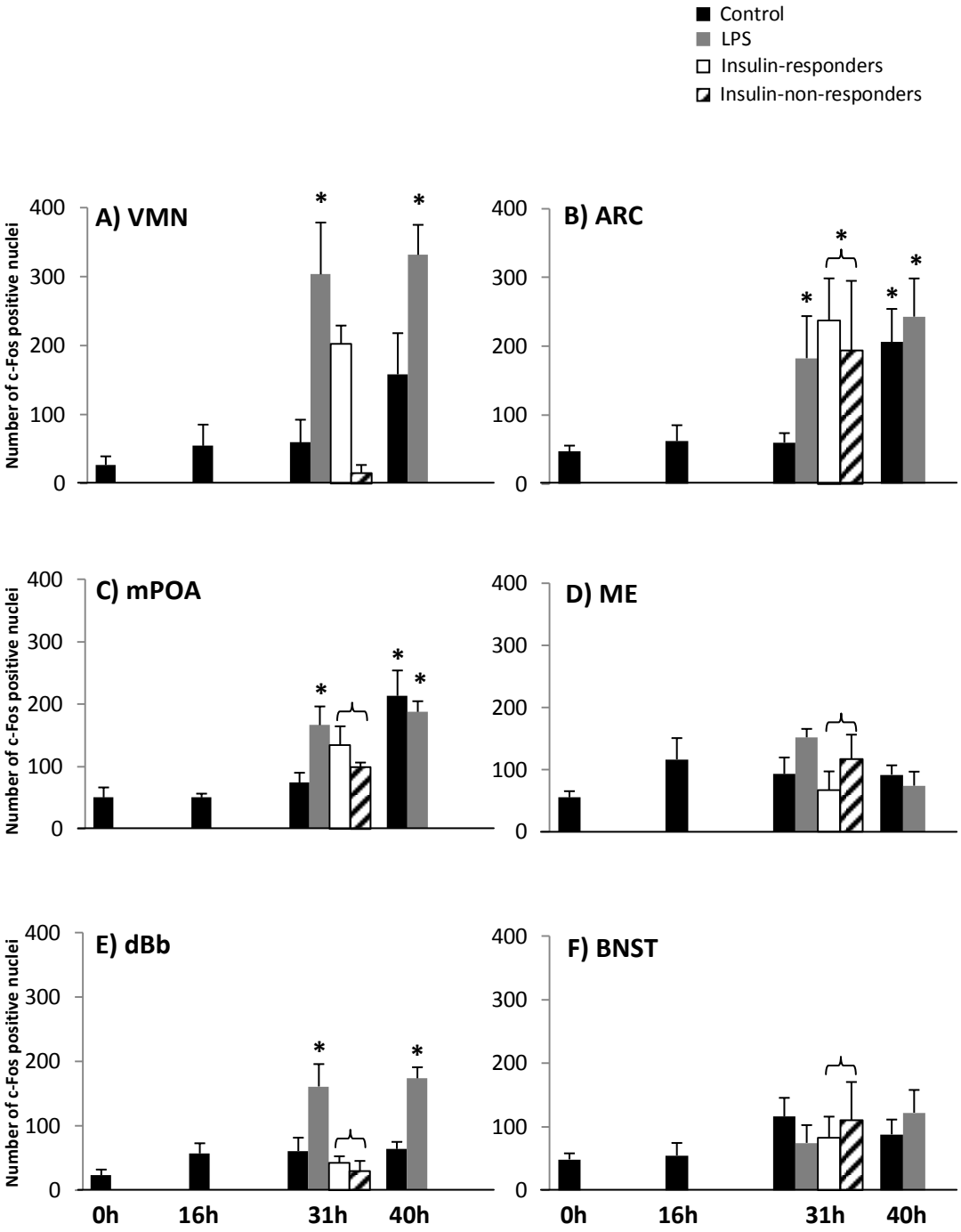

Fig. 5 Mean (±SEM) number of c-Fos positive nuclei in the A) VMN, B) ARC, C) mPOA, D) ME, E) dBb and F) BNST, at different times during the follicular phase of control and treated ewes. Animals are grouped according to killing time after PW, i.e., control ewes at 0h, 16h, 31h and 40h (n=4-5 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars) and insulin at 31h (insulin-responders, n=2; white bars and insulin-non-responders, n=2; hatched bars). Due to the split response in the VMN after insulin treatment, statistical analysis was not carried out and the data are presented for information. However, in the ARC, mPOA, ME, dBb and BNST, there were no split responses observed and therefore statistical analyses were carried out with both groups combined (n=4). Treatment with insulin or LPS was at 28h after PW. 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.

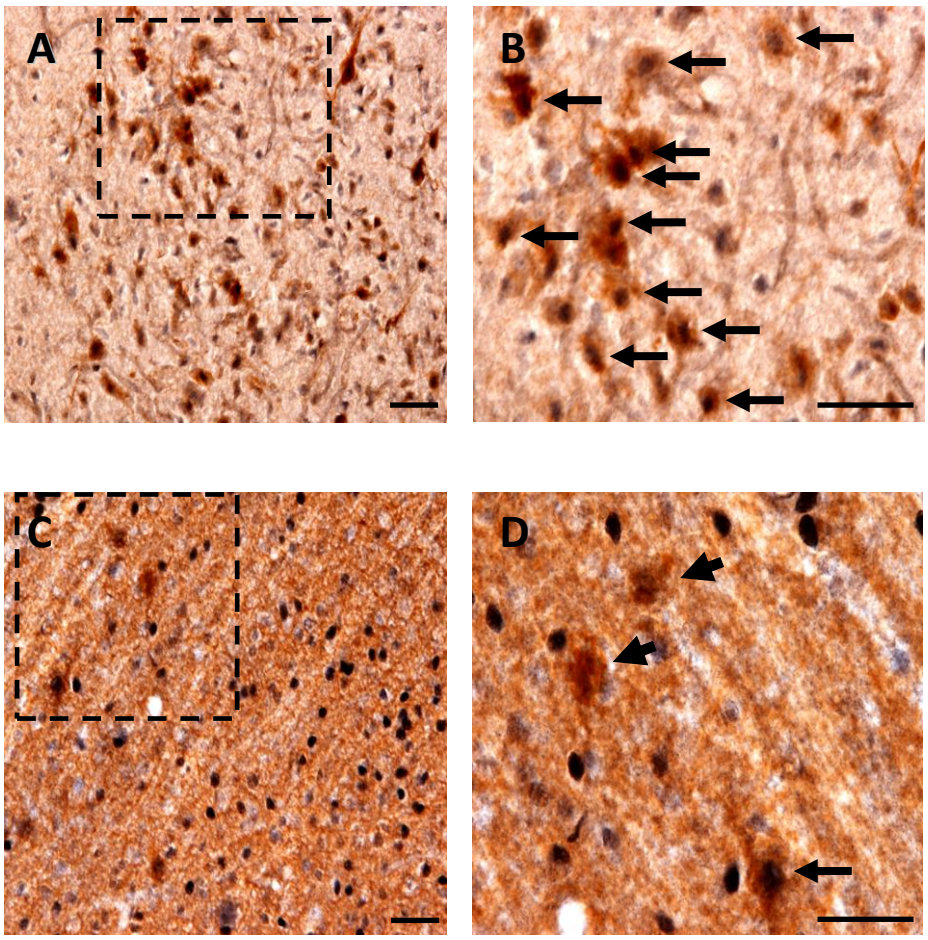

Fig. 6 Photomicrographs of the ARC nucleus that were dual-labeled for kisspeptin cells and their co-expression with c-Fos in control animals during the LH surge (A and B) as well as 12 h after LPS treatment in the late follicular phase (C and D). The *right panels* in each section are the higher magnifications (20 μm) of the *boxed areas* shown in the *left panels* (50 μm). *Black arrows* indicate examples of dual-labeled cells, and *arrowheads* indicate single-labeled kisspeptin-positive cells.

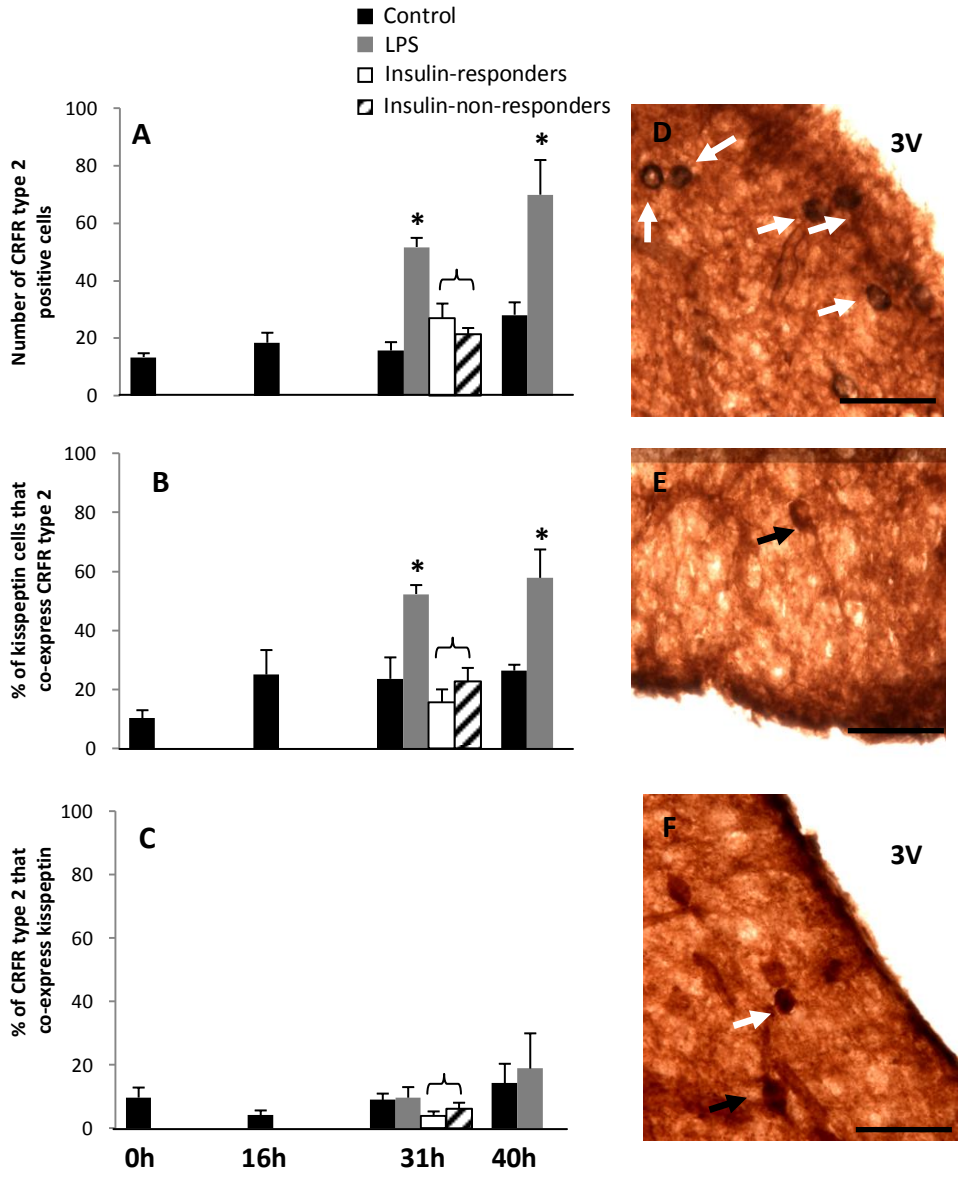

Fig. 7 (A) Mean (±SEM) number of CRFR type 2-positive cells in the lower part of the ARC and ME, (B) mean (±SEM) % number of kisspeptin cells that co-express CRFR type 2 and (C) mean (±SEM) % number of CRFR type 2-positive cells that co-express kisspeptin during the follicular phase of control and treated ewes. Animals are grouped according to killing time after PW i.e. control ewes at 0h, 16h, 31h and 40h (n=4-5 per group; black bars) as well as after insulin at 31h (insulin-responders, n=2; white bars and insulin-non-responders, n=2; hatched bars) and LPS at 31h and 40h (n=4 for both times; grey bars). There were no split responses observed in the insulin-treated animals and therefore, statistical analysis was carried out in both groups combined (n=4). Treatment with insulin or LPS was at 28h after PW. (D), (E), (F) Immunohistochemically identified CRFR type 2-positive cells and dual-labelled kisspeptin and CRFR type 2 cells in the lower part of the ARC and ME. *Black arrows* indicate examples of dual-labelled cells, and *white arrows* indicate single-labelled CRFR type 2 cells. *Scale bar*: 20μm. *P<0.05 compared to control and insulin sub-groups combined.

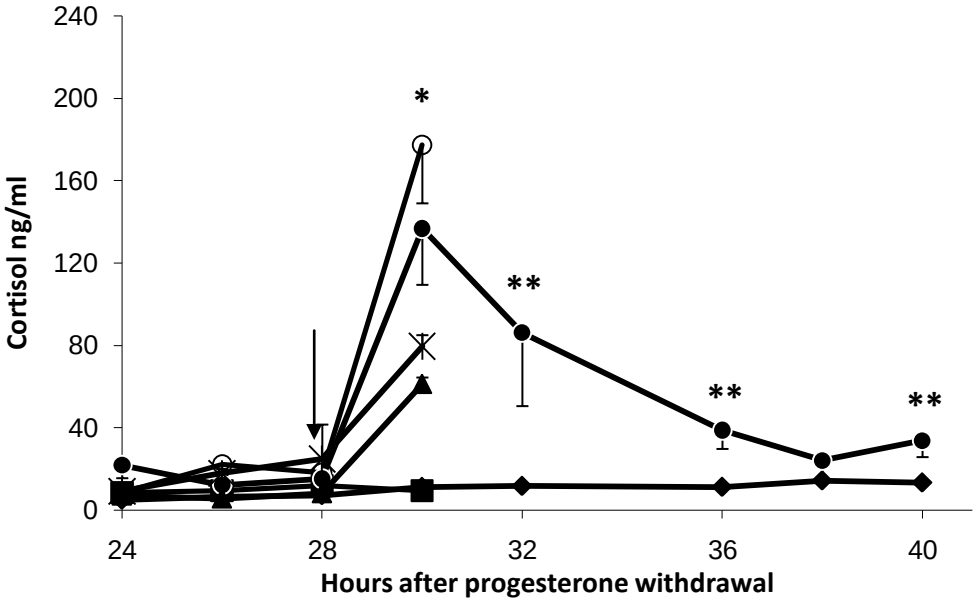

Fig. 8 Mean (±SEM) cortisol concentrations in 31h control (■; n=6), 31h insulin-responders (x; n=2), 31h insulin-non-responders (▲; n=2), 31h LPS (o; n=4), 40h control (♦; n=5) and 40h LPS (●; n=4) groups. There was no split response observed in the insulin-treated animals and therefore statistical analysis was carried out with both groups combined (n=4). The arrow indicates time of treatment. Some error bars are within the data symbols.

* 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).

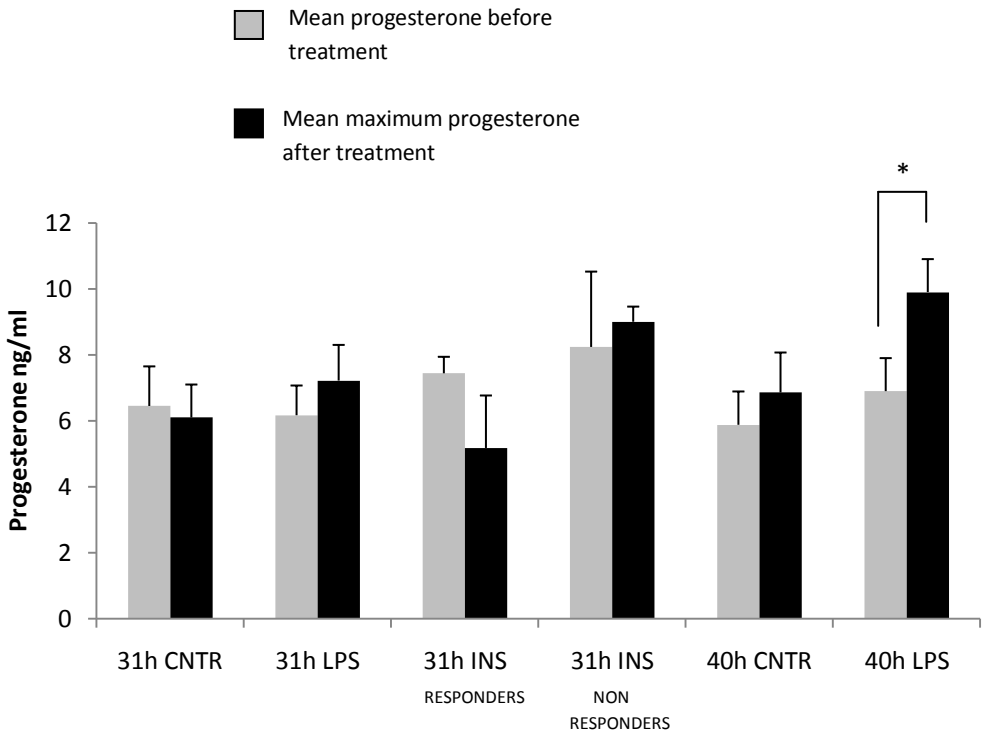

Fig. 9 Mean (±SEM) plasma progesterone concentrations at 28h after PW (before treatment; grey bars) and two mean (±SEM) consecutive maximum concentrations recorded after treatment (black bars), in the 31h control (31h CNTR; n=5), 31h LPS (n=4), 31h insulin-responders (31h INS-responders; n=2), 31h insulin-non-responders (31h INS-non-responders; n=2), 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).

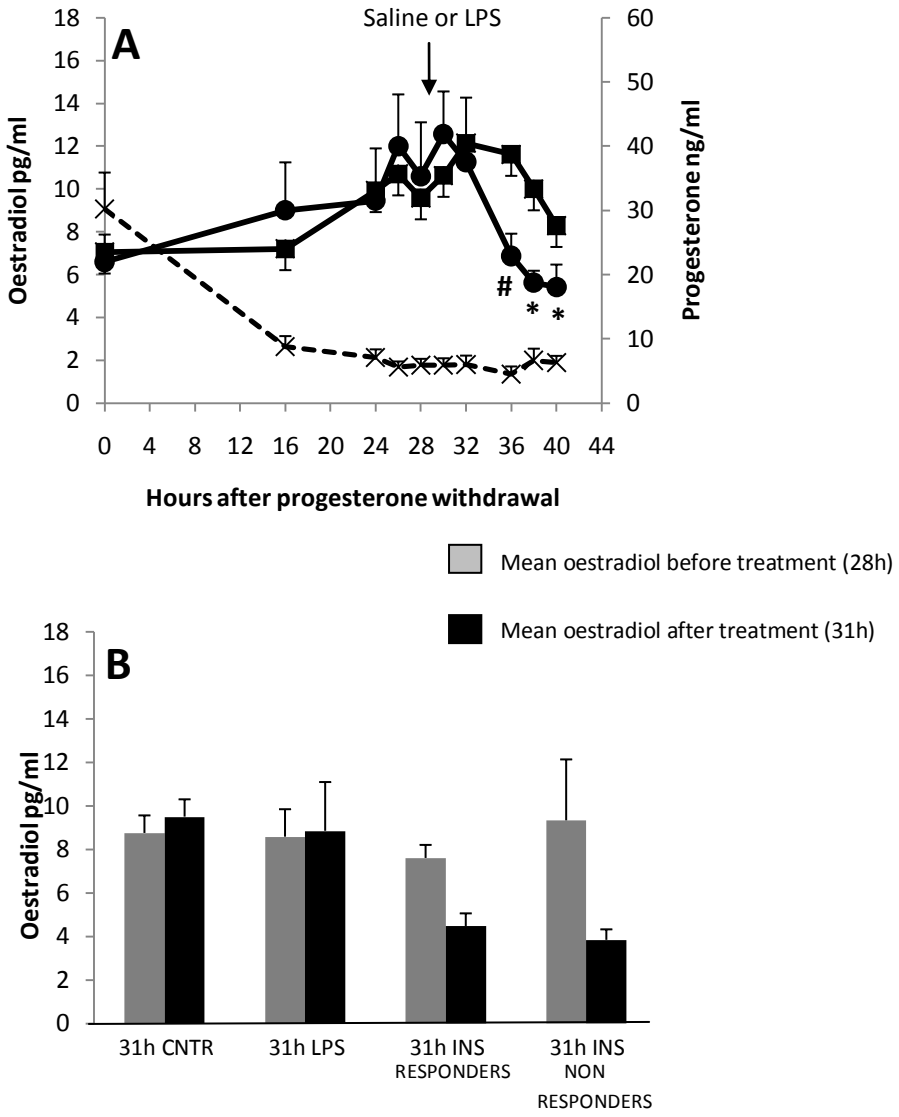

Fig. 10 A) Mean (±SEM) plasma oestradiol concentrations in 40h control (■; n=5) and 40h LPS (●; n=4) groups. Mean (±SEM) plasma progesterone concentrations in 40h control animals (×; n=5) during the follicular phase are also shown for comparison with oestradiol profiles. Progesterone concentrations after treatment are shown in Fig. 9. The arrow indicates the time of treatment. B) Mean (±SEM) plasma oestradiol concentrations at 28h after PW (i.e., before treatment; grey bars) and at 31h after PW (i.e., after treatment; black bars) in the 31h control (31h CNTR; n=5), 31h LPS (n=4), 31h insulin-responders (31h INS-responders; n=2), 31h insulin-non-responders (31h INS-non-responders; n=2). At 28h after PW oestradiol concentrations were not different between groups. # 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).

Table 1 Onset of the LH surge, pre-copulatory behaviour and oestrus (hours after PW) of individual ewes treated with saline or LPS or insulin at 28h after PW. Ewes were killed at 0h, 16h, 31h (31h control, 31h insulin 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					
31h INS					
Sheep O	23	27	27		
Sheep Q					
Sheep R					
Sheep S	29	29	29	29	
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	

Chapter 4

Chapter 4

Oestradiol receptor α and c-Fos co-expression in the medial preoptic area, arcuate nucleus and ventromedial nucleus at different times during the follicular phase of intact ewes and alteration after insulin or endotoxin

Keywords: c-Fos, oestradiol receptor alpha, stress, oestrous behaviour, LH surge, insulin, LPS, ewes.

Abstract

Oestradiol triggers the GnRH surge by activating ERα cells in the brain. The aim of the present study was to investigate the activation pattern of oestradiol receptor α (ERα) containing cells in the arcuate nucleus (ARC), ventromedial nucleus (VMN) and medial preoptic area (mPOA) during the follicular phase of intact ewes as well as determining whether stress-induced disruption of the LH surge involves a reduction of activated ERα-cells. Follicular phases of intact ewes were synchronised with progesterone vaginal pessaries. Control animals were killed at 0h, 16h, 31h and 40h (n=5-6 per group) after progesterone withdrawal (PW; time zero). At 28h, groups of animals received insulin (INS; 4 iu/kg) or endotoxin (LPS; 100 ng/kg) and were subsequently killed at 31h (INS; n=5 and LPS; n=5) or 40h (LPS; n=5). Hypothalamic sections were immunostained for ERα and a marker of neuronal transcriptional activation, c-Fos. LH surges occurred only in 40h control ewes: these animals had a marked increase in the percentage of ERα cells co-expressing c-Fos in the ARC (from 25 to 64%; $P<0.05$) and mPOA (from 17 to 40%; $P<0.05$) from 31h after PW and throughout the LH surge, as well as the VMN, but only during the surge (from 32 to 65%; $P<0.05$). However, when ewes were re-grouped according to behavioural status, there was a marked increase in the percentage of ERα-containing cells that co-expressed c-Fos in the VMN after the onset of sexual behaviour compared to before (from 5 to 57%; $P<0.000$). At 31h and 40h after PW (i.e., 3h and 12h after treatment, respectively), LPS decreased the percentage of ERα cells co-expressing c-Fos in the ARC (from 64 and 56% to 12 and 18%, respectively; $P<0.05$) and mPOA (from 40 and 62% to 11 and 13%, respectively; $P<0.05$), but there was no change in the VMN,

compared to controls. In contrast, at 31h after PW (i.e., 3h after treatment), insulin did not alter the percentage of ERα cells co-expressing c-Fos in the ARC compared to controls, whereas, two of four insulin-treated animals had a decrease in the mPOA (from 40 to 12%) and an increase in the VMN (from 32 to 78%) while two other had no increase (47 and 44%, for the mPOA and VMN, respectively). These results indicate that there is a specific temporal pattern of ERα-containing cell activation in the ARC, VMN and mPOA during the follicular phase of intact ewes and that this is disturbed by acute LPS or insulin administration in the late follicular phase.

Introduction

The ovarian steroid hormone oestradiol is of central importance in the control of reproductive neuroendocrine function in female mammals. For the greater part of the ovarian cycle in ewes, oestradiol and progesterone, act synergistically to restrain GnRH/LH (gonadotrophin releasing hormone/luteinising hormone) secretion through negative feedback action. However, during the late follicular phase, minute-by-minute portal blood sampling has revealed a 'switch' from inhibition to enhancement of GnRH secretion (Evans *et al.,* 1995, Karsch *et al.,* 1997). This constitutes oestradiol positive feedback and triggers the onsets of GnRH/LH surge secretion. However, steroid hormone signals do not impinge directly on GnRH cells as these cells do not possess progesterone receptors (PR) or oestradiol alpha receptors (ERα; Shivers *et al*., 1983, Herbison and Theodosis, 1992, Skinner *et al*., 2001). Some GnRH neurones express, ERβ (Hrabovszky *et al.,* 2001) although it is unlikely that ERβ plays a major role in the feedback regulation of GnRH/LH secretion, because ERβ knock-out mice have normal fertility (Lubahn *et al.,* 1993; Krege *et al.,* 1998). The surge generating mechanism has been well characterised in the ovariectomised (OVX) ewe (Evans *et al.,* 1997) and consists of three phases: i) activation, during which oestradiol concentrations reach a threshold and must remain elevated for a few hours (Moenter *et al.,* 1990, Caraty *et al.,* 2002). This signal is 'perceived' by neuronal cells that contain oestradiol receptors (ERα), and respond by becoming activated; ii) transmission, during which the activation signal is transmitted from ERα cells to GnRH neurones, either directly or via one or more interneurones; iii) surge secretion, during which there is a discharge of GnRH and LH. The latter two stages are hypothesised to be oestradiol independent (Evans *et al.,* 1997). The decrease in

plasma progesterone concentrations after luteolysis and the increase of oestradiol are also responsible for changes in sexual behaviour (Karsch *et al.,* 1980, Fabre-Nys and Martin 1991, Fabre-Nys and Gelez 2007). However, it remains to be elucidated whether a similar 'behaviour generating mechanism' exists.

The full phenotype and precise location of activated cells at each stage of the surge generating mechanism remain largely unknown. However, recent evidence has revealed that kisspeptin neurones, located in the medial preoptic area (mPOA) and arcuate nucleus (ARC), possess ERα (Franceschini *et al.,* 2006), and mediate most steroidal influences (negative and positive) on GnRH neurones (Smith, 2008, Roseweir and Millar 2009, Lehman *et al.,* 2010a, Caraty *et al.,* 2010). We have recently mapped the transcriptional activation of kisspeptin neurones in the mPOA and ARC, at various times in the follicular phase of intact ewes (Chapter 3), and found activation of kisspeptin cells to be low (consistent with negative feedback, Caraty *et al.,* 2010), except during the LH surge (i.e., surge secretion phase, consistent with positive feedback, Smith *et al.,* 2009). Thus, at least one other cell type is involved in the activation stage of the surge generating mechanism.

Sudden activation of the hypothalamus-pituitary-adrenal axis in the late follicular phase by the immunological stressor endotoxin (i.e., lipopolysaccharide; LPS) lowered plasma oestradiol concentrations and delayed the onsets of pre-copulatory behaviours, oestrus and the LH surge of free-running intact ewes; whereas, the metabolic stressor insulin-induced hypoglycaemia had the same effect in only 50% of animals (Fergani *et al.,* 2011). Furthermore, immunohistochemical analysis of c-Fos protein expression (a marker of neuronal transcription activation; Hoffman *et al.,* 1993) revealed that this disruption involved the activation of unknown cell types located in the VMN, ARC and mPOA (Chapter 3). In considering potential pathways by which stressors disrupt the follicular phase and sexual behaviour, it is important to note that there are four distinct mechanisms that may be involved: i) suppression of steroidogenesis at the ovarian level (Shakil *et al.,* 1994, Downing *et al.,* 1999, Battaglia *et al.,* 2000); ii) suppression of GnRH pulsatility (frequency or amplitude) from the hypothalamus (Battaglia *et al.,* 1997, Dobson and Smith, 2000); iii) suppression of LH pulsatile release from the pituitary (Williams *et al.,* 2001, Brothers *et al.,* 2010); and/or iv) prevention of the ability of the

surge-generating mechanism to respond to the preovulatory increases in plasma oestradiol concentrations (Battaglia *et al.,* 1999, Karsch *et al.,* 2002; Ghuman *et al*., 2011). The first three mechanisms could potentially deprive the ovarian follicle from the necessary gonadotrophin drive, thereby blocking the preovulatory oestradiol increase; however, the mechanism involved in surge disruption is not yet clear. This could involve inhibition of ERα-cell activation at appropriate times. Furthermore, there is evidence that the disrupting factors for each of these mechanisms (GnRH/LH pulse and surge secretion as well as steroidogenesis) may be different (Breen *et al.,* 2004).

In the present study, we examined brain tissue of intact ewes sacrificed at various times during the follicular phase with or without the administration of insulin or LPS. Our aim was to map the pattern of ERα transcriptional activation (by measuring colocalisation with c-Fos) in the ARC, VMN and mPOA, and correlate with peripheral plasma progesterone and oestradiol concentrations, as well as the exhibition of sexual behaviour and the LH surge. Furthermore, we sought to determine whether the disruption of the surge mechanism after LPS or insulin involves inhibition of ERα-cell activation in the ARC, VMN or mPOA as well as describing the temporal relationships between these changes and alterations in plasma steroid concentrations.

Materials and Methods

Animals, study design and blood sampling procedure

In this investigation we used tissue collected during the breeding season for an earlier study details of which are given in Chapter 3.

Visual observation of oestrous behaviour

Details are given in Chapter 3.

Tissue collection

Details are given in Chapter 3.

ERα and c-Fos dual-label immunofluorescence

For ERα/c-Fos analysis, three sections (40μm) from the mPOA (at the level of the organum vasculosum of the lamina terminalis (OVLT) and six from the middle and caudal

ARC (three from each level) were processed for dual-label immunofluorescence. All steps were performed at room temperature unless otherwise stated. Antibodies were diluted with 2.5% normal donkey serum (catalogue item S2170, Biosera, UK), 1% Triton X-100 (T9284, Sigma-Aldrich, UK) and 0.25% sodium azide (Sigma) in 0.1M phosphate buffer saline, pH 7.2 (PBS). Free-floating sections were washed thoroughly in PBS for 2h to remove the cryoprotectant solution followed by 1h incubation in blocking solution (10% donkey serum in PBS). This was followed by 72h incubation at 4^0C with a mixture of polyclonal rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA) at a dilution of 1:5000 along with monoclonal mouse anti-ERα (ID5, M7047, Dako, Carpinteria, CA, USA) at a dilution of 1:50. After incubation with the primary antisera, sections were washed thoroughly and incubated with a mixture of donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch, West Grove, PA) and donkey anti-mouse DyLight 488 (715-485-151, Jackson Immunoresearch, West Grove, PA) both diluted 1:500 for 2h. Thereafter, sections were washed with PBS followed by a final wash with double-distilled water, mounted on chrome alum gelatine coated slides and cover-slipped with Vectashield anti-fading mounting medium (Vector Laboratories Ltd, UK, H-1000). The c-Fos (Ghuman *et al.* 2010) and ERα (Skinner and Herbison, 1997) antibodies have been validated for the use in ovine neural tissue. In addition, negative controls that omitted one of the primary antibodies completely eliminated the appropriate fluorescence without obviously affecting the intensity of the other fluorescent probe.

Data analysis

Sections were examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20× objective. Photographs acquired with an image analysis program AxioVision (Zeiss Imaging Systems) and consisted of single c-Fos staining, single ERα staining as well as a merged image to produce a spectral combination of green (fluorescein) and red (rhodamine) that resulted in yellow-marked dual labeled cells. The areas examined were (as defined by Welento *et al.,* 1969): VMN (4 photographs per section, 2 sections per ewe), ARC (3 photographs per section, 3 sections per ewe, which consisted sections from the rostral, middle and caudal divisions of the nucleus) and mPOA (at the level of the OVLT, 2 photographs per section,

3 sections per ewe). Sections were evaluated unilaterally and each photograph was taken from a random field within each nucleus. All photographs were imported in to Image J version 1.42q, where counts were performed using the cell count plug-in. Initial counts were carried out on the merged image and ERα and c-Fos colocalisation was confirmed using side by side images of the individual ERα and c-Fos micrographs and visually identifying cells that contained both ERα label and c-Fos label with respect to microscopic tissue landmarks. 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. Mean (±SEM), as presented in figures and results, was calculated by averaging values for each group.

The data were analysed in two ways: the first consisted of control ewe data grouped according to time after PW, then by sexual behavioural status and whether an LH surge had occured; i.e., grouped into those killed at 0h and 16h after PW, those killed at 31h after PW but before the onset of sexual behaviour (Before behaviour, n=3), those killed at 31h or 40h after PW, after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). This grouping allowed for a detailed comparison of neuropeptide profiles in control animals at different stages of the follicular phase in our intact-ewe model. Secondly, control and treated animal data were grouped according to time of killing after PW, and this was used to compare treatment effects.

During data analysis, it became clear that there was a split response in the insulin group regarding the percentage of ERα-containing cells that co-expressed c-Fos in the mPOA and VMN. Therefore, this group was separated into two subgroups referred to hereafter as insulin-responders or insulin-non-responders (verified previously in Chapter 3 as with or without c-Fos activation in the paraventricular nucleus, respectively). As this division reduced the group size to n=2/group, statistical analysis was not undertaken, but the data are presented for information. When responses were not different between sub-groups, data were combined and analysed with n=4.

Regression analysis was used to examine the association between the percentage of change from 0h to the two mean consecutive lowest or highest plasma progesterone or oestradiol values, respectively, and the percentage of ERα cells that co-expressed c-Fos in each area in control animals.

Results

Luteinising hormone (LH) and behavioural profiles.

LH and behaviour profiles have been presented in detail in Chapter 3.

Control ewes: ERα and c-Fos *co-expression in the ARC, VMN and mPOA at different stages during the follicular phase, in animals grouped by behaviour.*

ARC

The percentage of ERα containing neurones that co-expressed c-Fos increased two-fold in the 'Before behaviour', 'After behaviour' and 'Surge' groups compared to 0h and 16h groups (P<0.05, for all comparisons; Fig. 1A).

mPOA

The percentage of ERα-containing neurones that co-expressed c-Fos sequentially increased from 0h towards 'Before behaviour', to 'After behaviour' as well as animals in the 'Surge' group (P<0.05, for all comparisons; Fig. 1C).

VMN

The percentage of ERα-containing neurones that co-expressed c-Fos gradually decreased from 0h towards the 'Before behaviour' group (P<0.05; Fig. 1E) and then suddenly increased ten-fold in 'After behaviour' and 'Surge' animals (P<0.05; Fig. 1E).

Oestradiol

Oestradiol data have been presented in Chapter 3. Using regression analysis, the percentage of ERα-containing cells that co-expressed c-Fos was positively associated with the percentage change in oestradiol concentration between 0h to the mean two consecutive highest plasma oestradiol values: in the mPOA (P=0.001, RSq=51.1%; Fig.

1D) and to a lesser extent in the VMN (P=0.02, RSq=21.1%; Fig. 1F) but not the ARC (P=0.7; Fig. 1B).

Progesterone

Progesterone data have been presented in Chapter 3. Furthermore, the percentage of ERα-containing cells that co-expressed c-Fos was associated with the percentage change in progesterone concentration between 0h to the mean two consecutive lowest plasma progesterone values: in the ARC (P=0.000, RSq=64. %; Fig. 1B) and the mPOA (P=0.000, RSq=51. %; Fig. 1D) but not the VMN (P=0.1; Fig. 1F).

Cortisol

Cortisol data have been presented in chapter 3.

Control, insulin or LPS: ERα and c-Fos co-expression in the ARC, VMN and mPOA at different times during the follicular phase, in animals grouped by hours after PW.

Photomicrographs of sections from the ARC and the mPOA dual-labelled for ERα and c-Fos are shown in Fig. 2 and Fig. 3, respectively. Mean numbers of c-Fos and ERα containing cells in the ARC, mPOA, and VMN, are given in Table 1.

ARC

With respect to PW, the percentage ERα containing cells that co-expressed c-Fos in controls was increased at 31h and remained high at 40h, the time of the LH surge (*P*<0.000 for both; compared to 0h and 16h control groups, Fig. 4A). However, at 31h after PW (i.e., 3h after LPS administration), there was a marked decrease in the percentage of ERα-containing neurones the co-expressed c-Fos in the LPS group (*P*<0.000) compared to controls (Fig. 4A). The effect of LPS was still evident between the control and LPS groups at 40h after PW, (i.e., 12h after the initial application of saline or LPS; *P*<0.000, Fig. 4). All insulin-treated animals (insulin-responders and insulin-non-responders combined; n=4) did not differ from controls, but were different compared to LPS-treated animals (*P*<0.05; Fig. 4A).

mPOA

With respect to PW, there was a gradual increase in co-expression with c-Fos, with 31h and 40h control groups having a higher percentage of ERα-containing cells that co-expressed c-Fos compared to 0h and 16h control groups (P<0.01; for all comparisons, Fig. 4B). However, at 31h after PW (i.e., 3h after insulin or LPS administration), there was a marked decrease in the percentage of ERα-containing neurones that co-expressed c-Fos in the LPS (P<0.05) and in the insulin-responders sub-group; Fig. 4B) but not in the other insulin-non-responders sub-group. Differences between the control and LPS treatments were still evident in the 40h groups, (i.e., 12h after the initial application of saline or LPS; P<0.000; Fig. 4B).

VMN

With respect to PW, the percentage of ERα-containing cells that co-expressed c-Fos was increased in control animals at 40h compared to 0h and 16h groups (P<0.02; Fig. 4C). Percentages in the 31h control group varied considerably between animals (see before) and therefore there was no difference from the rest of the control groups. However, at 31h after PW (i.e., 3h after insulin or LPS administration), there was a marked increase in the percentage of ERα-containing neurones that co-expressed c-Fos in the insulin-responders, but not in the 31h control, LPS, and the insulin-non-responder groups. At 40h after PW (i.e., 12h after the initial application of saline or LPS), the percentage of ERα-containing cells that co-expressed c-Fos increased in the 40h LPS group compared to the 31h LPS group (P<0.02) but were not different compared to the 40h control group.

Discussion

The present results extend our knowledge concerning the steroidal regulation of the GnRH/LH surge and sexual behaviour by the ARC, VMN and mPOA. We have demonstrated that the pattern of ERα cell activation changes during the follicular phase of intact ewes as well as between regions at specific time points. Furthermore, this pattern is disturbed by acute insulin or LPS administration in the late follicular phase, and the profile of disruption differs between the two stressors. This indicates a stressor type or intensity differential effect on ERα cell activation which, in both cases, leads to disruption of the LH surge.

Pattern of ERα activation during the follicular phase of intact ewes.

At 31h after PW (i.e., 1-2h before the expected onset of sexual behaviour and approximately 6-7 hours before the expected GnRH/LH surge onset), there was an marked increase in the percentage of activated ERα-containing neurones in the middle and caudal ARC, and this coincided with decreased progesterone and increasing oestradiol concentrations in plasma. This indicates that there are ERα-cells within this area that 'perceive' the steroid hormone milieu and respond by becoming transcriptionally activated. This is in accordance with the activation stage of the GnRH/LH surge mechanism. Furthermore, ERα activation was maintained throughout the late follicular phase and during the GnRH/LH surge, indicating that ERα cells in the ARC participate in the transmission and surge secretion phases of the GnRH surge mechanism. It has been suggested that the latter two stages are oestradiol independent, i.e., do not need a further or continuous oestradiol signal (Evans *et al.,* 1997, Harris *et al.,* 1998, Harris *et al.,* 1999), however, it appears that ERα neurones are still activated during this period. Even though, oestradiol concentrations in the present study were beginning to decrease as the LH surge occurred.

We have recently shown that the LH surge of intact ewes is accompanied by a simultaneous intense transcriptional activation of ARC kisspeptin neurones and not previously within the follicular phase (Fergani *et al.,* 2011). This indicates that kisspeptin cells are only involved in the surge secretion mechanism for GnRH surge release. Thus, it seems that kisspeptin cells are not solely responsible for the positive feedback effects of oestradiol as there are other cell types activated 6-7h before the expected surge onset, and these are not kisspeptin cells but contain ERα. In this respect, there are a number of different neurochemical phenotypes containing ERα in the ARC that have been discovered to date as potential candidates for this role. While the most striking accumulation of ERα is in kisspeptin neurones in the middle and caudal ARC of female sheep (Franceschini *et al.,* 2006), kisspeptin cells in this region, but not in the mPOA, co-localise two other neuropeptides important in the control of GnRH secretion: dynorphin and neurokinin B (Foradori *et al.,* 2006, Goodman *et al.,* 2007; Topaloglu *et al.,* 2009). This cell population has a very high degree (95%), of colocalisation with both ERα and PR (Franceschini *et al.,* 2006; Smith *et al.,* 2007) so could be critical for conveying the

respective positive and negative feedback influences of oestradiol and progesterone onto GnRH neurones (Lehman *et al.,* 2010b). As 94% of kisspeptin cells co-localise dynorphin and 80% co-localise neurokinin B, with an equally high reciprocal co-localisation (Goodman *et al.,* 2007), immunohistochemical detection of kisspeptin protein should reflect presence of all three neuropeptides. Thus, immunohistochemical analysis of dynorphin or neurokinin B with c-Fos would potentially produce a similar pattern to the one we observed in kisspeptin cells. However, in the ewe, kisspeptin and dynorphin immunoreactivity and/or gene expression fluctuate depending on hormonal and gonadal status (Foradori *et al.,* 2005, Smith *et al.,* 2007, Merkley *et al.,* 2009, Smith *et al.,* 2009, Smith 2009). It is, therefore, probable that the transcriptional activation of ERα cells during the activation phase of the GnRH surge may reflect neurokinin B or dynorphin activation.

Other potential cell types involved are β-endorphin, tyrosine hydroxylase (TH) a marker for dopamine, neuropeptide Y (NPY) or somatostatin (Antonopoulos *et al.,* 1989) and a relatively small percentage of these possess ERα (3% to 20%; Lehman and Karsch 1993, Skinner and Herbison 1997, Scanlan *et al.,* 2003). In addition, 52-61% glutamatergic neurones in the ARC/VMN express ERα, are activated by oestradiol, and project to the mPOA where most GnRH neurones are located (Pompolo *et al.,* 2003b). Tourlet *et al.,* (2005) also suggest a role for galanin in oestradiol regulation with over 50% galanin neurones containing ERα across the ovine hypothalamus and POA. Cells containing all the above neuropeptides have been implicated in the control of GnRH secretion in the ewe (Anderson *et al.,* 2001, Scalan *et al.,* 2003, Advis *et al.,* 2003, Pompolo *et al.,* 2003a, Tourlet *et al.,* 2005 Taylor *et al.,* 2007) and are, therefore, potential candidates for mediating the stimulatory effect of steroids on GnRH secretion. There are additional candidates containing other potential neuropeptides, also with ERα and located in the ARC, although their role has not yet been confirmed in the ewe, for example, neurotensin examined in rats (Antonopoulos *et al.,* 1989, Alexander, 1993, Herbison, 1998) and GABA in the monkey (Thind and Goldsmith, 1997).

The positive feedback actions of oestradiol are likely to be exerted within the vicinity of the ER-expressing cells of the VMN to evoke the GnRH surge and sexual behaviour in the ewe (Blache *et al.,* 1991, Caraty *et al.,* 1998). However, after the discovery of kisspeptin

and its importance in regulating steroid feedback, the role of the VMN in the surge initiation mechanism has been under debate, as this area contains very few kisspeptin cells (Franchessini *et al.,* 2006). Nevertheless, the VMN is particularly rich in ER (Lehman *et al.,* 1993, Herbison *et al.,* 1993, Blache *et al.,* 1994,) and activation after oestradiol, especially in the ventrolateral aspect, is sexually dimorphic (Robinson *et al.,* 2010). In the present study, the percentage of activated ERα containing neurones in the VMN gradually decreased from 0h towards the mid-follicular phase, before the onset of sexual behaviour, followed by an intense activation in animals that exhibited behaviour and an LH surge. This initial gradual down-regulation coincided with the decrease in plasma progesterone. Indeed, progesterone acts as a priming hormone in the control of sexual behaviour by increasing the number of ER in the VMN during the luteal phase, thereby increasing sensitivity to oestradiol (Blache *et al.,* 1994). By analogy, this initial decrease of active ERα-neurones in the VMN may reflect decreasing progesterone concentrations. Interestingly, ERα activated neurones then increased ten-fold in animals exhibiting pre-copulatory behaviours/oestrus implying that ER containing cells in the VMN may be involved in oestradiol stimulation of sexual behaviour. Indeed, ER in the ventrolateral part of the VMN has been identified as the most sensitive site for oestradiol action on sexual behaviour in the female rat (Rubin and Barfield, 1980, Rissman *et al.,* 1997, Spiteri *et al.,* 2009), sheep (Blache *et al.,* 1991, Blache *et al.,* 1994) and monkey (Michael *et al.,* 2005).

The neurochemical identity of cell types located in the VMN of sheep are poorly defined. However, 35% of somatostatin cells in the VMN contain ER, which accounts for 70% of the total ER immunoreactive cells in this area (Herbison, 1995). Furthermore, these cells are activated in a sex-specific manner by short-term exposure to oestradiol (Robinson *et al.,* 2010). Therefore, somatostatin may be involved in oestradiol actions on oestrous behaviour although this needs further investigation. To date dopamine and noradrenaline have received most attention and are major regulators of sexual behaviour (Fabre-Nys *et al.,* 1994, Fabre-Nys and Gelez 2007). Indeed, dopaminergic neurones originating from the ARC send projections towards the VMN (Qi *et al.,* 2008) which co-localises dopamine receptor 1 in sheep (Colthorpe and Curlewis, 1996). In the rat, noradrenergic receptors 1b increase in the VMN after oestradiol treatment (Etgen

and Morales, 2002). The origin of these noradrenergic fibres are the arousal-related neuronal groups A1 and A2 in the rat brainstem (Kow *et al*. 1992). Relative to this, in the ewe, noradrenaline increases transiently in mediobasal hypothalamus (MBH) extra-cellular fluid during oestrus and following sexual interactions with a male (Fabre-Nys *et al.,* 1997). It would be of great interest to determine whether somatostatin cells receive input from dopamine and/or noradrenaline, constituting a possible mechanism for the control of sexual behaviour in the ewe. Cells containing galanin and glutamate are also located in the VMN and contain ERα but their role in oestrous behaviour has not yet been investigated in the ewe (Tourlet *et al.,* 2005; Pompolo *et al.,* 2003a).

Whether the mPOA is involved in positive or negative feedback in the ewe is controversial. Oestradiol implants in this vicinity elicited negative feedback but not positive feedback (Caraty *et al.,* 1998). However, a recent study, using an intact but steroid-treated ewe model, showed that mPOA kisspeptin cells are transcriptionally activated during the surge and, therefore, implicated in the positive feedback process (Hoffman *et al.,* 2010). Similarly, Smith *et al.,* (2009) found an increase in kisspeptin mRNA in the mPOA at the time of the LH surge. We have also recently shown that ~50% of kisspeptin cells are activated at the time of the LH surge in the intact ewe indicating that the mPOA participates in positive feedback by oestradiol, and is specifically associated with the surge secretion phase but not signal recognition or transmission (Chapter 3). In the present study, the percentage of ERα containing cells that were transcriptionally activated in the mPOA gradually increased culminating in peak activation during the surge. Interestingly, we observed the same gradual escalation of kisspeptin neurone activation as with ERα (Chapter 3).

Apart from the 50% of kisspeptin cells in the ovine mPOA that contain ERα, other potential candidates are the 40% of the gamma aminobutyric acid synthesising cells that contain ERα (GABA; Herbison *et al.,* 1993). Indeed, microdialysis revealed decreased GABA levels in the mPOA prior to the surge (Robinson *et al.,* 1991). Furthermore, nearly all dynorphin cells in this area contain PR (Foradori *et al.,* 2002) and therefore ERα (Dufourny and Skinner, 2002). In addition, over 50% of galanin-containing-cells also contain ERα and may contribute to the escalating activation of the mPOA (Tourlet *et al.,* 2005).

Our present results demonstrate that oestradiol and progesterone have different region and time specific effects on activation of ERα containing cells. In the ARC, the activational pattern of ERα cells was correlated with circulating plasma progesterone concentrations but not oestradiol. Thus, it appears ERα-cells within this area are not activated by oestradiol in a dose-dependent manner but may rather 'perceive' a threshold of oestradiol, and respond by becoming transcriptionally active (Caraty *et al.,* 2002, Saifullizam *et al.,* 2010). Furthermore, this requires low concentrations of progesterone. In contrast, the VMN activational pattern had a very low level of correlation with plasma oestradiol and none to progesterone. We, therefore, speculate that the VMN is activated subsequent to the initial activation of the ARC, consistent with the transmission phase of the surge generating mechanism. However, increased activation of ERα neurones in this area coincides with the occurrence of sexual behaviours providing evidence that this area could be involved in the sexual behaviour mechanism. Nonetheless, it appears that the ARC is the site to initially respond to an oestradiol signal of sufficiently high or increasing levels (Moenter *et al.,* 1990). Interestingly, in the mPOA, there was a simple linear relationship between circulating plasma oestradiol and progesterone concentrations and the percentage of ERα neurones that were transcriptionally activated, indicating that the mPOA is regulated by ovarian steroids in a dose-dependent manner. The reason for this differential regulation is not known, however, it appears that the ARC, VMN and mPOA participate in oestradiol positive feedback, but are controlled by ovarian steroids in different ways.

Pattern of ERα activation during the follicular phase of intact ewes treated with LPS or insulin.

Plasma oestradiol concentrations decreased 8h after the administration of LPS, whereas a decrease in the percentage of activated ERα neurones, in the ARC and mPOA, occurred sooner (3h after treatment). These results concur with previous studies indicating that there are at least two mechanisms involved in LPS inhibition of the ovarian cycle: one involving disruption of GnRH/LH pulses and, therefore, reduced oestradiol secretion; and the other preventing the ability of the surge-generating mechanism to respond to the preovulatory increase in oestradiol (Battaglia *et al.,* 1999, Karsch and Battaglia, 2002). Here, we extend these observations by showing that the latter mechanism involves

suppression of activated ERα-containing cells in the ARC and mPOA. Furthermore, our results show that there is a time difference between the two disruptive mechanisms, indicating that the regulating factors may be different. In support of this, Harris *et al.,* (2000) report that prostaglandins secreted after LPS have the ability to attenuate GnRH pulses, but administration of the prostaglandin synthesis inhibitor flurbiprofen cannot reverse the LH surge delay observed after this stressor (Breen *et al.,* 2004). Cortisol is known to suppress pulsatile LH secretion in a dose-dependent fashion (Debus *et al.,* 2002). However, in the present study, cortisol increased to maximum concentrations immediately after the administration of LPS (i.e., 2h after treatment) and is, therefore, a potential candidate for the immediate inhibition of ERα-containing neurones. In accordance, Pierce *et al.,* (2009) and Wagenmaker *et al.,* (2009a) report that cortisol disrupts the positive feedback effect of oestradiol to trigger an LH surge. In addition, ~ 70% of ERα cells co-express glucocorticoid receptors type II (GRII; the mPOA and ARC (Dufourny and Skinner, 2002). We observed an increase in plasma progesterone concentrations after LPS, however, the timing of maximum values varied considerably between animals, from 2h to 10h after treatment and, therefore, we cannot determine which mechanism is affected by LPS-induced increases in progesterone. It is noteworthy that progesterone has been implicated in both inhibition of GnRH pulses (Karsch *et al.,* 1987) and blocking the surge mechanism (Kasa-Vubu *et al.,* 1992, Skinner *et al.,* 1998, Richter *et al.,* 2002, Smith *et al.,* 2003, Richter *et al.,* 2005). The potential influence of pyrexia, remains to be determined, however, our previous studies showed that maximum body temperatures after the same dose of LPS occurred 4h after treatment (Fergani *et al.,* 2011) indicating that pyrexia may contribute to the attenuation of GnRH pulses, presumably via prostaglandin. The effects of LPS were still evident 12h after treatment, when the percentage of activated ERα neurones remained at low levels. Taking in to consideration that these animals did not have an LH surge at the same time as controls, we can conclude that the inhibition of ERα neurone activation is a major contributing factor to the LH surge disruption in response to an immune/inflammatory challenge in the ewe. This compliments our recent results in which the absence of an LH surge was accompanied by a failure of highly oestradiol-receptive kisspeptin neurones to be activated (Chapter 3).

Intriguingly, the percentage of ERα neurones that were transcriptionally activated was not altered in the VMN by LPS, even though the total number of c-Fos positive cells in this area increased (Chapter 3). A possible explanation for this is that the VMN contains very few GRII (Dufourny and Skinner, 2002) and is not directly influenced by cortisol. Somatostatin cells in the VMN contain ERα (70% of the total ER immunoreactive cells in this area; Herbison, 1995), therefore, we speculate that at least some of the ERα neurones in the VMN activated after LPS are, indeed, somatostatin in phenotype. Another possible cell type could be ERα containing inhibitory GABA cells which are abundant in the rat VMN (Luine *et al.* 1997).

Interestingly, there was a split response in the insulin-treated animals with two out of four having an intense increase in the VMN, and a concurrent decrease in the mPOA, of the percentage of activated ERα neurones 3h after treatment (insulin-responders), whereas the remaining two ewes were similar to controls (insulin-non-responders). An equivalent divergence was observed in our previous studies when 10 out of 20 animals treated with insulin did not have a delay in sexual behaviour or the LH surge (insulin-non-delayed subgroup; Fergani *et al.,* 2011) and did not display an intense transcriptional activation in the PVN and VMN (insulin-non-responders; Chapter 3). This discrepancy is often observed after insulin administration (Fergani *et al.,* 2011, Chapter 3). The reason for this is not known, however, the only observed hormonal difference between the two groups of animals was a subtle increase in plasma progesterone (Fergani *et al.,* 2011). In contrast, the percentage of activated ERα neurones 3h after treatment in the ARC increased in both insulin subgroups. This concurs with our recent findings that acute insulin administration in the late follicular phase immediately increases the number of activated kisspeptin cells in the ARC (Chapter 3). Therefore, the increased percentage of activated ERα neurones observed in the present study may be, at least in part, kisspeptin cells.

Plasma oestradiol concentrations began to decrease immediately after insulin treatment and this coincided with the increase in activated ERα cells in the ARC and VMN but a decrease in the mPOA. The reason for the time lag in oestradiol decrease between insulin and LPS groups is not known. However, hypoglycaemia is induced immediately after insulin administration and is considered to be the cause of GnRH/LH pulse and

surge inhibition leading to disruption of the surge mechanism (Dobson and Smith 2000, Smith *et al.,* 2003). Indeed the GnRH pulse and surge generator is particularly sensitive to reduced glucose concentrations in rats (Kawaguchi *et al.,* 1998). However, the immediate decrease of plasma oestradiol concentrations may reflect a quicker inhibition of steroidogenesis in the ovary after insulin compared to LPS (Shakil *et al.,* 1994, Downing *et al.,* 1999, Battaglia *et al.,* 2000).

Plasma cortisol concentrations increased simultaneously in both insulin sub-groups as observed in our earlier study (Fergani *et al.,* 2011), in which cortisol concentrations increased after insulin treatment in animals whether the LH surge was delayed or not, indicating that cortisol alone is not responsible for LH surge disruption after insulin. In support of this, the insulin-induced LH surge delay is not reversed by the progestin/glucorticoid receptor antagonist RU486 (Dobson and Smith 2000). Interestingly, Wagenmaker *et al.,* (2009b) report similar findings after the application of a layered psychosocial stress paradigm, i.e., the stressor appears to have a central effect by attenuating GnRH pulses but this is not reversed by RU486, indicating that cortisol was not the mediator. It is possible that insulin-induced hypoglycaemia and psychosocial stress are less intense stressors than the dose of LPS used and, therefore, cortisol production is not sufficient enough to have a hypothalamic effect. Indeed in the present study after LPS or following infusion of cortisol to interfere with the LH surge (Wagenmaker *et al.,* 2009b), plasma cortisol concentrations reached a mean maximum of 157 and 172ng/ml, respectively. In contrast, after insulin-induced hypoglycaemia and psychosocial stress, maximum cortisol concentrations were much lower, i.e., 70 and 57 ng/ml, respectively.

In conclusion, the present findings clearly show that the ERα activational pattern differs during the follicular phase of intact ewes, as well as between regions at specific time points. Based on our observations, we hypothesise that once circulating progesterone concentrations have decreased and oestradiol concentrations reach a specific 'threshold' (approximately 6-7h before the expected LH surge onset) ERα cells in the ARC become transcriptionally active. ERα cells in the VMN are activated later than the ARC, and this coincides with the exhibition of sexual behaviours supporting existing evidence that the VMN is involved in behavioural regulation. In the mPOA, ERα cells followed a different

temporal pattern and were activated gradually. Nonetheless, ERα cell activation was maximum during the LH surge in all these areas, indicating a role in oestradiol positive feedback and GnRH surge secretion. However, this activational pattern was disturbed by acute insulin or LPS administration in the late follicular phase. Interestingly, LPS inhibited the activation of ERα cells in the ARC and mPOA but not the VMN, whereas insulin stimulated activation in the ARC and the VMN and inhibited the mPOA (the latter two in half of the animals). Thus, disruption of the LH surge by acute stressors involves stimulation and/or inhibition of ERα neurone activation and this varies between stress type or intensity, but in both cases may lead to the disruption of the LH surge.

Acknowledgements

Thanks are due to Nigel Jones and the farm staff for care of the animals; Hilary Purcell, David Jones and Peter Taylor for technical assistance; and Prof A Parlow and NIAMDD, USA for LH standard preparations. We are also grateful to Richard Morris for his guidance in immunohistochemical techniques and Dr. Michael Morris for help with animal observations.

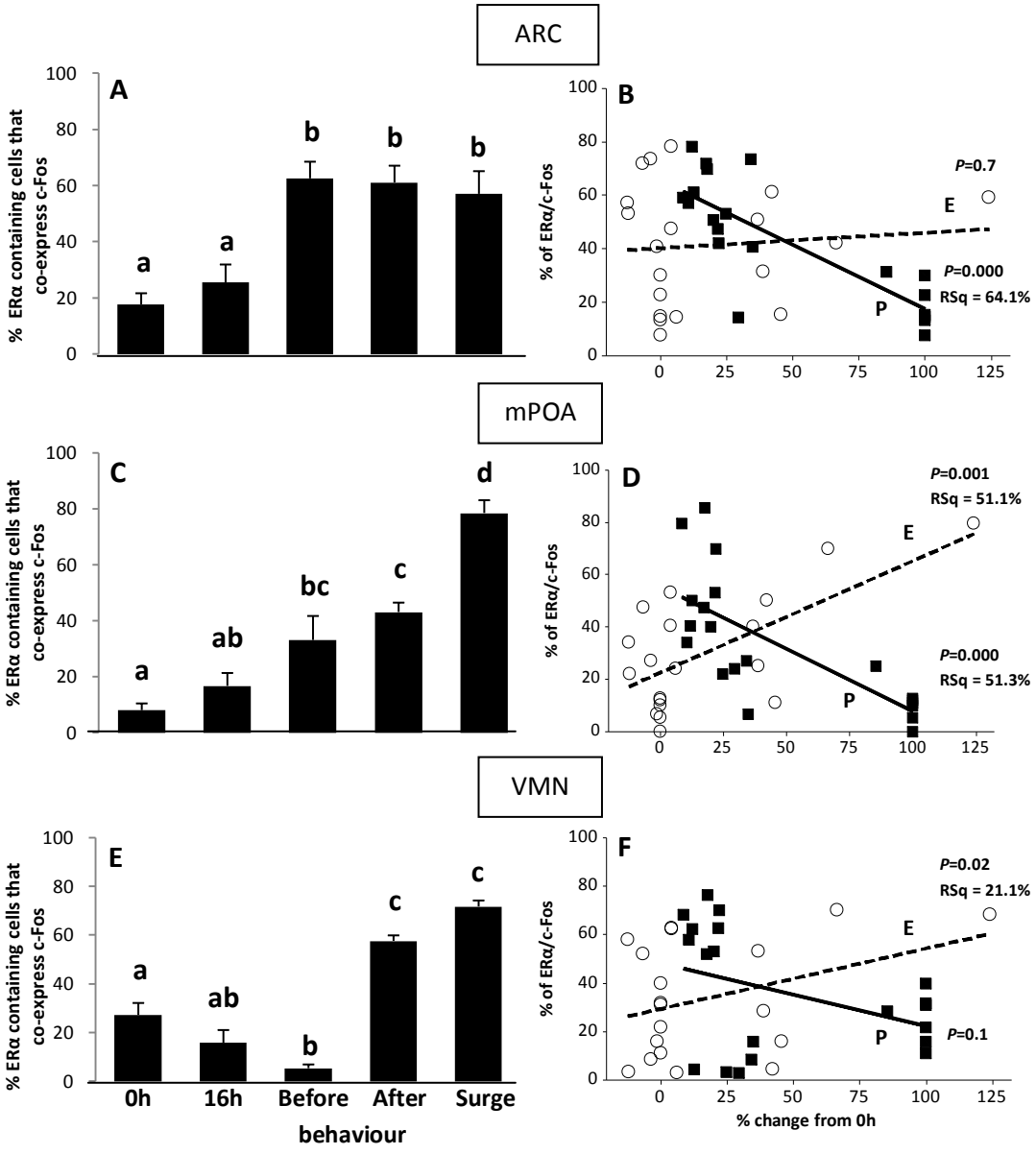

Fig 1. A,C,E: Mean % (±SEM) of ERα containing cells that co-expressed c-Fos in the ARC, mPOA and VMN at different stages during the follicular phase of control ewes. Animals are grouped according to time as well as hormonal and behavioural status; i.e., grouped into those killed at 0h and 16h after PW (n=4-5), those killed before the onset of sexual behaviour (Before behaviour, n=3), those killed after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the 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). B,D,F: Regression graphs showing the correlation between the % of ERα containing cells that co-express c-Fos (% ERα/c-Fos) in the ARC, mPOA and VMN against the % change from 0h to the mean two consecutive highest or lowest concentrations for oestradiol (○, E; dotted line) or progesterone (■, P; solid line), respectively.

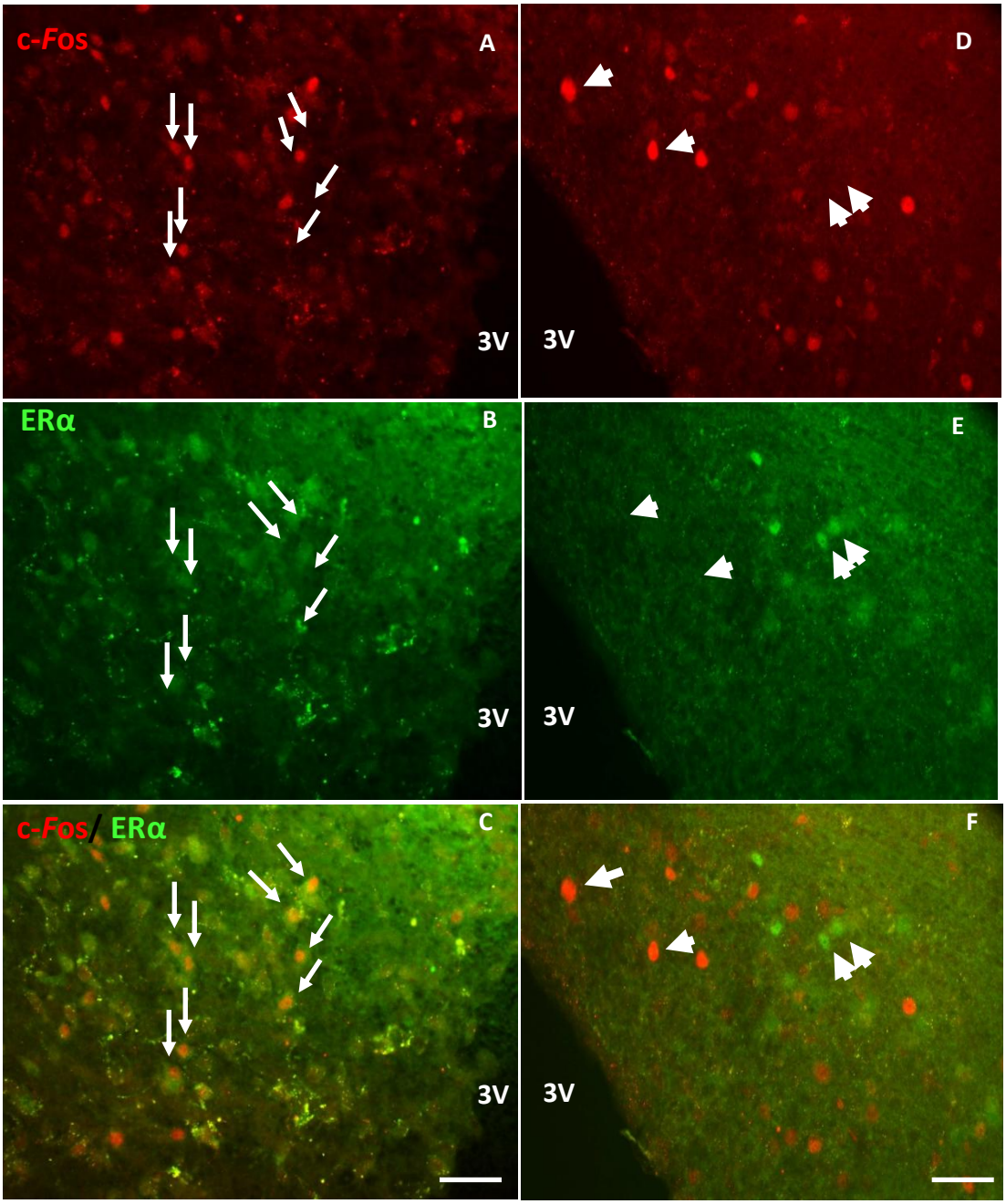

Fig. 2 Sets of photomicrographs from the ARC nucleus that were dual-labelled for c-Fos containing cells (A,D) and their co-expression with ERα (B,E) in control animals at 31 h after PW (A, B, C) as well as 3 h after LPS treatment in the late follicular phase (D, E, F). Panels on the bottom (C, F) are computer-generated merged images of the two top panels illustrating co-expression of c-Fos and ERα. Examples of single and double labelled cells are marked through the panels with arrowheads and arrows, respectively. *Scale bars* = 20 μm. 3V = third ventricle.

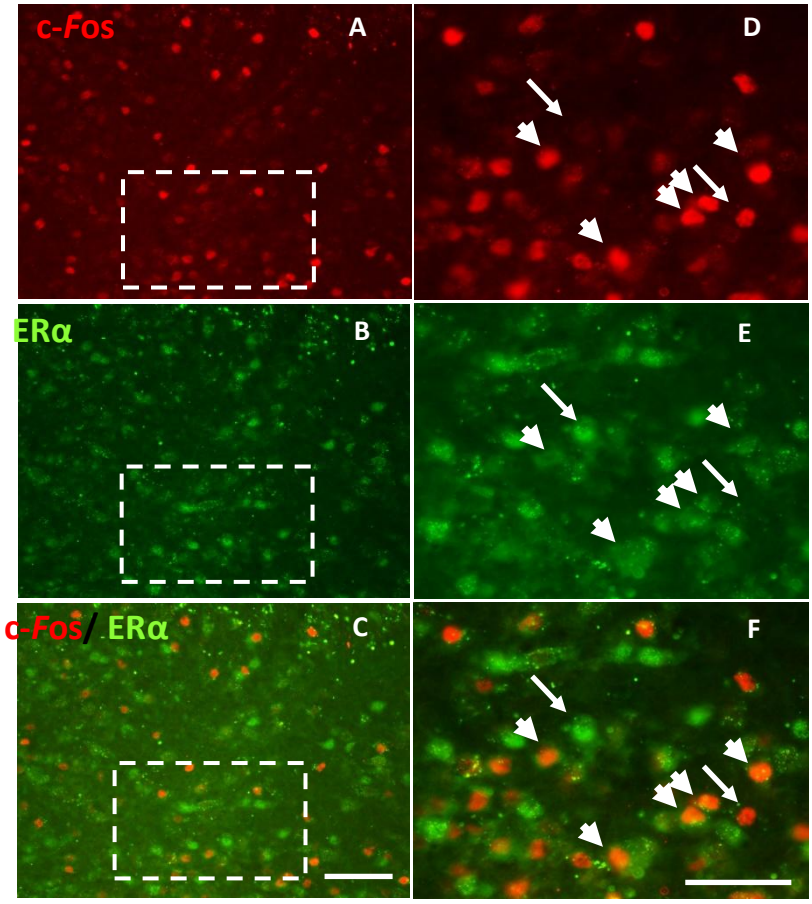

Fig. 3 Sets of photomicrographs from the mPOA that were dual-labelled for c-Fos containing cells (A,D) and their co-expression with ERα (B, E) in control animals at 40 h after PW (during the surge; A, B, C). Panels on the bottom (C, F) are computer-generated merged images of the two top panels illustrating co-expression of c-Fos and ERα. The right *panels* (D, E, F) are the higher magnifications (Scale bar: 50 μm) of the *boxed areas* shown in the *left panels* (A, B, C; scale bar: 20 μm). Examples of single- and double-labelled cells are marked through the panels with arrows and arrowheads, respectively.

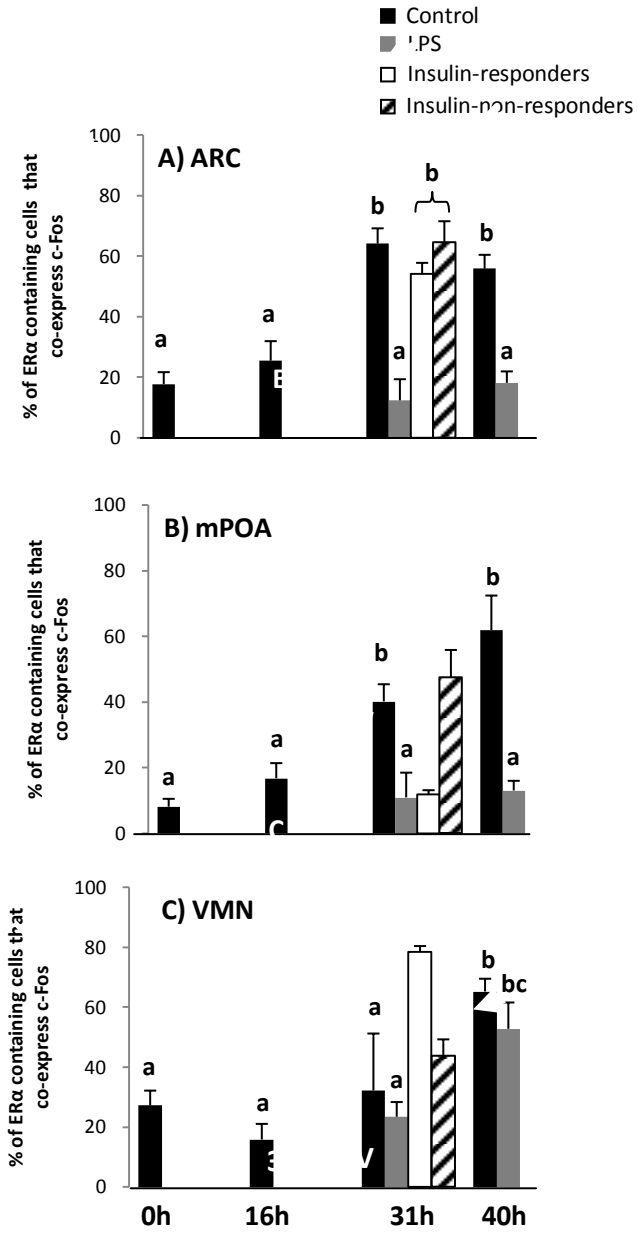

Fig 4. Mean (±SEM) % of ERα containing cells that co-express c-Fos in the ARC, VMN and mPOA at different times during the follicular phase of control and treated ewes. Animals are grouped according to killing time after PW i.e. control ewes at 0h, 16h, 31h and 40h (n=4-5 per group; black bars) as well as after insulin at 31h (insulin-responders, n=2; white bars and insulin-non-responders, n=2; hatched bars) and LPS at 31h and 40h (n=4 for both times; grey bars). Due to the split response in the mPOA and VMN after insulin treatment, statistical analysis was not carried out and the data are presented for information. However, in the ARC, there were no split responses observed and, therefore, statistical analysis was carried out with both groups combined (n=4). Treatment with insulin or LPS was at 28h after PW. Within each panel, differences between percentages are indicated by different letters on top of each bar ($P<0.05$).

Table 1 Mean (± SEM) numbers of c-Fos and ERα containing cells in the ARC, mPOA, and VMN

Group	Region					
	No of c-Fos positive cells			No of ERα positive cells		
	ARC	mPOA	VMN	ARC	mPOA	VMN
0 h	86,1 ± 19,4	45,8 ± 6,6	65,2 ± 4.0	52,3 ± 26,9	15,2 ± 4,1	38,0 ± 8,34
16 h	131,4 ± 22,9	58,3 ± 17,9	83,5 ± 24,3	57,9 ± 19,5	17,2 ± 3,4	49,8 ± 16,4
31 h control	171,5 ± 26,5	79,0 ± 18,1	96,0 ± 28,2	96,6 ± 21,1	39,5 ± 13,0	49,6 ± 14,4
31h LPS	185,8 ± 15,9	161,4 ± 15,2*	211,5 ± 34,6*	41,3 ± 16,6	37,1 ± 22,5	78,0 ± 12,9
31h insulin-responders	226,5 ± 12,0	90,0 ± 18,5	199,0 ± 21,0	79,2 ± 2,2	32,5 ± 13,5	139,0 ± 24,0
31h insulin-non-responders	259,3 ± 47,3	143,8 ± 10,8	75,5 ± 10,5	156,8 ± 77,3	81,5 ± 22,5	72,0 ± 9,0
40h control	156,5 ± 19,1	95,7 ± 23,4	179,4 ± 33,3*	89,7 ± 19,3	59,0 ± 26,2	100,4 ± 20,8*
40 h LPS	147,9 ± 32,3	120,4 ± 28,5	203,3 ± 12,7*	59,9 ± 18,2	26,0 ± 6,6	75,3 ± 12,7

ARC c-Fos: * $P<0.05$ compared to 0h control groups

mPOA c-Fos: * $P<0.05$ compared control groups.

VMN c-Fos: * $P<0.05$ compared to 0h, 16h and 31h control groups.

VMN ERα: * $P<0.05$ compared to 0h, 16h, 31h control groups.

Chapter 5

Chapter 5

Kisspeptin, dynorphin and neurokinin B co-expression with c-Fos in the middle and caudal ARC at different times during the follicular phase of intact ewes and alteration after insulin or endotoxin.

Keywords: Kisspeptin, dynorphin, neurokinin B, c-Fos, ewe, insulin, LPS, stress, GnRH surge, LH surge.

Abstract

The aim of the present study was to determine the activation pattern of kisspeptin, neurokinin B and dynorphin (KNDy) cells in the middle and caudal ARC during the follicular phase of intact ewes, as well as investigating whether patterns are altered by stressors. Follicular phases of intact ewes were synchronised with progesterone vaginal pessaries. Control animals were killed at 0, 16, 31 and 40h (n=5-6 per group) after progesterone withdrawal (PW; time zero). At 28h, groups of animals received insulin (4 iu/kg) or endotoxin (LPS; 100 ng/kg) and were subsequently killed at 31h (insulin; n=5 and LPS; n=5) or 40h (LPS; n=5). LH surges occurred only in 40h control ewes. Activation patterns were established using c-Fos (a marker for neuronal activation) with dual- and triple-label immunohistochemistry. In control ewes, the maximum percentage of dynorphin cells co-expressing c-Fos was observed at 31h after PW and before the onset of sexual behaviour (52 and 56%, respectively; $P<0.05$, compared to 0h and 16h control groups), whereas the maximum kisspeptin and neurokinin B cells co-expressing c-Fos occurred at 40h after PW (49 and 42%, respectively; $P<0.05$ for both) specifically in animals exhibiting an LH surge (64 and 50%, respectively; $P<0.05$, compared to all other control groups). Furthermore, at 31h after PW, the number of cells containing dynorphin increased compared to kisspeptin cells (59.9 ± 6.2 *versus* 37.2 ± 3.0 total number of cells; $P<0.05$). However, LPS decreased the percentage of dynorphin cells co-expressing c-Fos (at 31h and 40h after PW; to 23% and 11%, respectively; $P<0.05$) and kisspeptin cells (at 40h after PW; to 22%; $P<0.05$). In contrast, at 31h after PW (i.e., 3h after treatment), insulin decreased the percentage of dynorphin cells co-expressing c-Fos in two of the insulin-treated animals (to 21 and 33%; insulin-responders) but not in the other two; whereas the percentage of activated kisspeptin cells increased in all insulin-treated

animals (52%; $P<0.05$). Neurokinin B was not altered by either treatment. These results indicate that KNDy cells are involved in the GnRH/LH surge mechanism and its disruption after LPS or insulin involves alteration of kisspeptin and dynorphin (but not neurokinin B) cell activation.

Introduction

The ovarian steroid hormone milieu varies during the oestrous cycle and involves positive and negative feedback on gonadotrophin-releasing hormone (GnRH) neurones. The decrease in progesterone concentrations after luteolysis along with an increase of oestradiol is responsible for the sudden and massive release of GnRH that evokes the pituitary luteinizing hormone (LH) surge, responsible for ovulation in mammals. However, the majority of studies indicate that GnRH neurones have very few, if any, receptors for ovarian steroids (Herbison *et al.* 1993, Lehman and Karsch 1993, Sullivan *et al.* 1995, Scott *et al.* 2000, Skinner *et al.* 2001). Therefore, these hormones exert modulatory effects mainly through intermediate neurones containing steroid receptors (Herbison 1998, Herbison *et al.* 2001, Hrabovszky *et al.* 2001, Smith and Jennes 2001). Recent evidence has revealed that the hypothalamic neuropeptide kisspeptin mediates steroidal influence on GnRH neurones across a large number of species (Smith 2008, Roseweir and Millar 2009; Lehman *et al.* 2010; Chapter 3). Furthermore, nearly all kisspeptin cells in the arcuate nucleus (ARC; specifically the middle and caudal aspects), but not the preoptic area (POA), co-localise two other neuropeptides that are important in the control of GnRH secretion: neurokinin B and dynorphin (Foradori *et al.,* 2006, Goodman *et al.,* 2007, Topaloglu *et al.,* 2009, Lehman *et al.,* 2010a). Thus, this ARC cell group co-localises neuropeptides that are both stimulatory (kisspeptin) and inhibitory (dynorphin) to GnRH secretion (Pillon *et al.,* 2003, Dungan *et al.,* 2006; Kauffman *et al.,* 2007, Lehman *et al.,* 2010b) and are thus called KNDy cells (co-localising kisspeptin, neurokinin B and dynorphin; Cheng *et al.,* 2009, Lehman *et al.,* 2010a). Furthermore, 95% of KNDy cells possess oestradiol receptor α (ERα; Franceschini *et al.,* 2006) and progesterone receptors (PR; Faradori *et al.,* 2002) indicating that they may mediate both positive and negative feedback effects of ovarian steroids on GnRH secretion. To date

KNDy cells have not been associated with the control of oestrous behaviour (Kauffman *et al.* 2007).

We have recently shown that activation of kisspeptin immunoreactive cells (assessed by the co-expression with c-Fos) increases intensely in the rostral, middle and caudal ARC during the LH surge compared to other stages in the follicular phase (Chapter 3). As 94% kisspeptin cells co-localise dynorphin and 80% co-localise neurokinin B, with an equally high reciprocal co-localisation (Goodman *et al.,* 2007), immunohistochemical detection of kisspeptin protein may reflect the presence of all three neuropeptides. Thus, immunohistochemical analysis of dynorphin or neurokinin B with c-Fos might produce an identical pattern to that observed in kisspeptin cells. However, in the ewe, kisspeptin and dynorphin immunoreactivity and/or gene expression fluctuates depending on hormonal and gonadal status (Faradori *et al.,* 2005, Smith *et al.,* 2007, Merkley *et al.,* 2009, Smith *et al.,* 2009, Smith 2009). Therefore, we hypothesise that, in an intact ewe model, endogenous fluctuation of the ovarian steroid hormone milieu during the follicular phase is associated with variation in protein expression and, therefore, different activation patterns for each neuropeptide cell phenotype.

There is evidence that KNDy neurones may mediate the effects of stressors on the reproductive neuroendocrine axis. Indeed, a few studies report down-regulation of the hypothalamic kisspeptin system in rats and male rhesus monkeys after metabolic or immune/inflammatory stressors, such as negative energy balance (Castellano *et al.* 2010), short term fasting (Wahab *et al*. 2010) or administration of E. coli endotoxin (LPS; Iwasa *et al.* 2008, Kinsey-Jones *et al.* 2009). Furthermore, each of these stressors was accompanied by suppression of the hypothalamic-pituitary-gonad axis. We have recently shown that a sudden activation of the hypothalamus-pituitary-adrenal axis by LPS in the late follicular phase lowered plasma oestradiol concentrations and delayed onsets of pre-copulatory behaviours, oestrus and the LH surge of free-running intact ewes; whereas, insulin-induced hypoglycaemic stress had the same effect in only 50% of animals (Fergani *et al.,* 2011). Furthermore, immunohistochemical analysis of kisspeptin or ERα combined with c-Fos revealed that this disruption after LPS or insulin is

respectively accompanied by inhibited or stimulated activation of ERα and kisspeptin cells in the ARC (Chapter 3, Chapter 4). However, the effects of stressors on dynorphin and neurokinin B immunoreactivity and activation in the ARC have not yet been investigated.

In the present study, we examined brain tissue of intact ewes sacrificed at various times during the follicular phase with or without the administration of insulin or LPS. Our aims were to: 1) map the pattern of kisspeptin, dynorphin and neurokinin B transcriptional activation (by measuring co-expression with c-Fos; Hoffman *et al.,* 1993) in the middle and caudal ARC, where most of these neurones are located; 2) correlate changes in these patterns with contemporaneous plasma progesterone and oestradiol concentrations, as well as signs of sexual behaviour and the LH surge; 3) compare co-expression between kisspeptin and dynorphin using triple label immunohistochemistry (kisspeptin/dynorphin/c-Fos); and 4) establish whether immunoreactivity and/or co-expression vary during the follicular phase. Finally, we sought to determine whether the disruption of the surge mechanism after LPS or insulin involves inhibition of kisspeptin, dynorphin and/or neurokinin B activation in the middle and caudal ARC, as well as describing the temporal relationships between these changes and alterations in plasma steroid concentrations.

Materials and Methods

Animals, study design and blood sampling procedure

In this study, we used tissue collected during the breeding season; details are given in Chapter 3.

Visual observation of oestrous behaviour

Details are given in Chapter 3.

Tissue collection

Details are given in Chapter 3.

c-Fos and dynorphin or neurokinin B dual-label immunofluorescence

c-Fos/dynorphin or c-Fos/neurokinin B dual-label immunofluorescence was carried out on 40μm sections containing middle and caudal levels of the ARC (three sections for each level and neuropeptide combination). 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) in 0.1M phosphate buffer saline, pH 7.2 (PBS). Free-floating sections were washed thoroughly in PBS for 2h to remove the cryoprotectant solution followed by 1h incubation in blocking solution (10% donkey serum in PBS). This was followed by 72h incubation at 4^0C with polyclonal rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA) at a dilution of 1:5,000. After incubation with the primary antiserum, sections were washed thoroughly and incubated with donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch, West Grove, PA) diluted 1:500 for 2h. A second immunofluorescent procedure was then performed, as described above, to localise the second primary antibodies: rabbit anti-dynorphin A serum (T-4268, Peninsula Laboratories, LLC, San Carlos, CA), at a dilution of 1:10000 or rabbit-anti-neurokinin B serum (T-4450, Peninsula Laboratories, LLC, San Carlos, CA), at a dilution of 1:1,000, both incubated for 72h at 4^0C and then visualised using donkey-anti-rabbit Dylight 488 (715-485-151, Jackson Immunoresearch West Grove, PA) at a dilution of 1:500. Thereafter, sections were washed with PBS followed by a final wash with double-distilled water, mounted on chrome alum gelatine-coated slides and cover-slipped with Vectashield anti-fading mounting medium (H-1000, Vector Laboratories Ltd, UK). The c-Fos (Ghuman *et al.* 2010), dynorphin A and neurokinin B (Faradori *et al.,* 2006, Goodman *et al.,* 2007) antibodies have been validated previously for use in ovine neural tissue. In addition, negative controls omitting one of the primary antibodies completely eliminated the appropriate fluorescence without obviously affecting the intensity of the other fluorescent probe.

c-Fos, dynorphin and kisspeptin triple-label immunohistochemistry

c-Fos/dynorphin/kisspeptin triple-label immunohistochemistry was carried out on sections containing middle and caudal levels of the ARC (three sections for each level). This consisted of an immunoperoxidase protocol in which nuclear c-Fos was detected first with diaminobenzidine as chromogen (DAB; brown reaction product) followed by

visualisation of dynorphin and kisspeptin with immunofluorescence. As dynorphin and kisspeptin antibodies were both derived in the rabbit, we used a previously described modified protocol (Hunyady *et al.,* 1996, Goodman *et al.,* 2007, Cheng *et al.,* 2010). In brief, the first antigen was visualised using a very low concentration of primary antibody with tyramide amplification solution (TSA). Next, the second antigen was visualised using normal concentrations of primary antibody and detection with fluorophore-conjugated secondary antibody. The immunohistochemical procedure was carried out at room temperature unless otherwise stated. Washes with PBS were performed after every step, except after blocking with donkey serum. Antibodies were diluted as described above. 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. Sections were then incubated for 1h in blocking solution (10% donkey serum in PBS), followed by a 72h incubation in rabbit anti-c-Fos antibody at a dilution of 1:5,000 at 4^0C. After incubation with primary antiserum, sections were labeled with biotinylated donkey anti-rabbit IgG (711-065-152, Jackson Immunoresearch West Grove, PA) at 1:500 for 2h, followed by 90 min in Vectastain Elite ABC kit (1:250 in PBS; PK6100, Vector Laboratories Ltd, UK). Nuclear c-Fos was visualised by 5 min incubation in DAB (SK-4100, Vector Laboratories, Ltd, UK). The second immunohistochemical procedure consisted of blocking with H_2O_2 and donkey serum (as described above) and incubation for 72h in rabbit anti-kisspeptin (lot 564; gift from Prof. Alain Caraty, INRA Nouzilly, France) at a dilution of 1:150,000 at 4^0C. Following incubation, sections were labeled with biotinylated donkey anti-rabbit IgG (1:500; for 2h) and then incubated in Vectastain Elite ABC kit (1:250; for 90 min). The signal was amplified in TSA for 10 min (1:200; New England Nuclear Life Science Products Life Sciences, Boston, MA) diluted in PBS with 0.003% H_2O_2 as substrate (Cheng *et al.,* 2010) and then labeled with streptavidin conjugated AlexaFluor 488 (S11223, Molecular Probes, Eugene, OR, USA) at a dilution of 1:100 for 2h. A third immunohistochemical procedure was then performed and consisted of blocking (as described above) followed by a 72h incubation with rabbit anti-dynorphin diluted 1:10,000 and subsequent labeling with donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch West Grove, PA) at a dilution of 1:500 for 2h. Finally, sections were washed, mounted on chrome alum gelatine-coated slides, dried, and

cover-slipped with Vectashield anti-fading mounting medium (H-1000; Vector Laboratories Ltd, UK). Negative controls included omission of either primary antibody and resulted in a complete lack of staining for the corresponding peptide**.** Slides were stored covered at 4^0C until analysed.

Data collection and analysis

Dual-labeled sections were examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20× objective. Photographs (acquired with an image analysis program AxioVision; Zeiss Imaging Systems) consisted of single c-Fos staining, single dynorphin or neurokinin B staining as well as a merged image to produce a spectral combination of green (fluorescein) and red (rhodamine). Triple-labeled sections were similarly examined for single c-Fos staining, single dynorphin and single kisspeptin as well as a merged image of the two latter to produce a spectral combination of green (fluorescein) and red (rhodamine). Sections were evaluated unilaterally and each of three photographs per section was taken from a random field within each area/section. 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-, dual- or triple-labeled 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 analysed in two ways: the first consisted of control ewe data grouped according to time as well as hormonal and sexual behavioural status; i.e., grouped into those killed at 0h and 16h after PW, those killed at 31h after PW but before the onset of sexual behaviour (Before behaviour, n=3), those killed at 31h or 40h after PW, after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). This grouping allowed for a detailed comparison of neuropeptide profiles in

control animals at different stages of the follicular phase in our intact-ewe model. Secondly, control and treated animals data were grouped according to time of killing after PW, and this was used to compare treatment effects.

During data analysis, it became clear that there was a split response in the insulin group regarding the percentage of dynorphin cells that co-expressed c-Fos in the ARC. Therefore, this group was separated into two subgroups referred to hereafter as insulin-responders or insulin-non-responders (verified previously in Chapter 3 as with or without c-Fos activation in the paraventricular nucleus, respectively). As this division reduced the group size to n=2/group, statistical analysis was not undertaken, but the data are presented for information. When responses were not different between sub-groups, data were combined and analysed with n=4.

Peripheral plasma progesterone and oestradiol values fluctuated between animals, thus, regression analysis was used to examine the association between the percentage of change from 0h to the two mean consecutive lowest or highest progesterone or oestradiol values, respectively, and the percentage of kisspeptin, dynorphin and neurokinin B cells that co-expressed c-Fos (i.e., were activated) in the ARC of control animals.

Results

Luteinising hormone (LH) and behavioural profiles.

Shown in chapter 3

Control ewes: Dynorphin and c-Fos co-expression in the middle and caudal ARC at different stages during the follicular phase, in animals grouped by behaviour (dual immunohistochemical study).

The percentage of dynorphin cells that co-expressed c-Fos was greatest in the 'Before behaviour' group compared to earlier stages in the follicular phase ($P<0.05$; Fig. 1A). Thereafter, there was a gradual decrease until the LH surge (Fig. 1A).

Control ewes: Kisspeptin, dynorphin and c-Fos co-expression in the middle and caudal ARC at different stages during the follicular phase in animals grouped by behaviour (triple immunohistochemical study).

The percentage of kisspeptin cells that co-expressed c-Fos sequentially increased from 0h to 'Before behaviour', and to 'After behaviour' ($P<0.05$ for all comparisons; Fig. 2A). Animals in the 'Surge' group displayed a two-fold increase in the percentage of kisspeptin cells that co-expressed c-Fos compared to the 'After behaviour' group ($P<0.05$; Fig. 2A). In contrast, there was a two-fold increase in the percentage of dynorphin cells that co-expressed c-Fos in the 'Before behaviour' group, compared to 0h and 16h ($P<0.05$ for both; Fig. 2C). This increase was maintained 'After behaviour' but not in the 'Surge' group (Fig. 2C). The pattern of changes in the proportion of cells co-expressing dynorphin and c-Fos was similar visualised by dual- or triple-staining (Figs. 1A and 2C).

The number of immunoreactive cells, as well as the percentage co-expression between kisspeptin and dynorphin cells, in the middle and caudal ARC, varied during the follicular phase (Fig. 3). In summary, the number of kisspeptin or dynorphin cells was greater 'After behaviour' compared to 0h, or 0h and 16h, respectively but, there were more dynorphin cells compared to kisspeptin cells 'Before behaviour' ($P<0.05$ for all comparisons; Fig. 3A). The percentage of kisspeptin cells co-expressing dynorphin was lower in the 'Surge' group (i.e., when there were more single-labelled kisspeptin cells) compared to 16h, 'Before behaviour' and 'After behaviour' ($P<0.05$ for all comparisons; Fig. 3B), whereas there were fewer dynorphin cells co-expressing kisspeptin (i.e., there were more single labelled dynorphin cells) 'Before behaviour' and 'After behaviour' compared to 0h and 16h ($P<0.05$ for all comparisons; Fig. 3B).

*Control ewes: Neurokinin B and c-*Fos *co-expression in the middle and caudal ARC at different stages during the follicular phase in animals grouped by behaviour (dual immunohistochemical study).*

The percentage of neurokinin B cells that co-expressed c-Fos sequentially increased from 0h to 16h and thereon to 'Before behaviour', 'After behaviour' as well as animals in the 'Surge' group ($P<0.05$ for 'Surge' compared to other stages; Fig. 2E). The number of cells with neurokinin B immunoreactivity was greater in the 'After behaviour' and 'Surge'

groups compared to all other stages in the follicular phase (P<0.05 for all comparisons; Fig. 3C).

Oestradiol

Oestradiol data have been presented in Chapter 3. Using regression analysis, the percentages of kisspeptin or neurokinin B cells that co-expressed c-Fos were positively associated with the percentage change in concentration from 0h to the mean two consecutive highest plasma oestradiol values in the middle and caudal ARC (P=0.005, RSq=36% and P=0.002, RSq=41% for kisspeptin and neurokinin B, respectively). This was not the case for dynorphin cells (P=0.5; Fig. 2B, 2D, 2F).

Progesterone

Progesterone data have been presented in chapter 3. Again using regression analysis, the percentages of kisspeptin, dynorphin or neurokinin B cells that co-expressed c-Fos were positively associated with the percentage change in concentration from 0h to the mean two consecutive lowest plasma progesterone values in the middle and caudal ARC (P=0.002 RSq=41%, P=0.001 RSq=47% and P=0.001 RSq=50% for kisspeptin, dynorphin and neurokinin B, respectively; Fig. 2B, 2D, 2F).

Cortisol

Cortisol data have been presented in Chapter 3.

Control, insulin or LPS: Dynorphin and c-Fos co-expression in the middle and caudal ARC at different times during the follicular phase, in animals grouped by hours after PW (dual immunohistochemical study).

With respect to PW, the percentage of dynorphin cells that co-expressed c-Fos in controls was increased at 31h compared to all other times examined in the follicular phase (P<0.0001; Fig. 1B). However, 31h after PW (i.e., 3h after LPS or insulin administration), there was a marked decrease in the percentage of dynorphin cells that co-expressed c-Fos in the LPS groups (P<0.05, Fig. 1B) and the insulin-responders (Fig. 1B), but not in the insulin-non-responders, compared to 31h controls (Fig. 1B).

Control, insulin or LPS: Kisspeptin, dynorphin and c-Fos co-expression in the middle and caudal ARC at different times during the follicular phase, in animals grouped by hours after PW (triple immunohistochemical study).

With respect to PW, the percentage of kisspeptin cells that co-expressed c-Fos in controls was increased at 31h and 40h compared to all other times examined in the follicular phase (Fig. 4A). However, 31h after PW (i.e., 3h after insulin or LPS administration), the percentage of kisspeptin cells that co-expressed c-Fos increased in both insulin sub-groups (from 32.8 ± 4.3 to 51.8 ± 4.8%; combined insulin-responders and insulin-non-responders; $P<0.05$; Fig. 4A), whereas the LPS group was not different to controls (Fig. 4A). At 40h after PW (i.e., 12h after LPS administration), the percentage of kisspeptin cells that co-expressed c-Fos was markedly lower in LPS treated animals compared to controls ($P<0.05$; Fig. 4A).

The percentage of dynorphin cells that co-expressed c-Fos in controls was increased at 31h and at 40h after PW compared to 0h and 16h control groups ($P<0.05$; Fig. 4B). However, at 31h i.e., 3h after insulin or LPS administration, the percentage of dynorphin cells that co-expressed c-Fos was lower in the LPS group ($P<0.05$) and in the insulin-responders (Fig. 4B), but not the insulin-non-responders. At 40h this was still evident in the LPS group i.e., 12h after LPS administration ($P<0.05$; Fig. 4B). The patterns of changes in the proportion of cells co-expressing dynorphin and c-Fos were similar visualised by dual- or triple-staining (Figs. 1B and 4B). Photomicrographs from the ARC triple-labelled with kisspeptin, dynorphin and c-Fos are shown in Fig. 5.

The number of immunoreactive cells is given in Tables 1 and 2, as well as the percentage co-expression between kisspeptin and dynorphin cells, in the middle and caudal ARC, during the follicular phase or after LPS or insulin treatment. In summary, the numbers of kisspeptin cells were greater in 31h and 40h control groups compared to 0h, whereas dynorphin cell number increased in the 31h compared to 0h and 16h control groups and at 40h compared to the 0h control group. In addition, there were more immunoreactive dynorphin cells than immunoreactive kisspeptin or neurokinin B cells in the 31h control groups (Table 1). Treatment with LPS decreased the number of cells with dynorphin immunoreactivity, whereas insulin decreased both kisspeptin and dynorphin immunoreactivity at 31h compared to the control group (Table 1).

Control, insulin or LPS: Neurokinin B and c-Fos co-expression in the middle and caudal ARC at different times during the follicular phase, in animals grouped by hours after PW (dual immunohistochemical study).

The percentage of activated neurokinin B cells in controls was greatest at 40h compared to 0h and 16h control groups ($P<0.05$, Fig.4C). LPS or insulin administration had no effect on the percentage of activated neurokinin B cells at any time after treatment (Fig. 4C). The number of neurokinin B immunoreactive cells did not vary during the follicular phase, or after LPS or insulin treatment, with respect to PW (Table 1). Photomicrographs of sections from the ARC dual-labeled with neurokinin B and c-Fos are shown in Fig. 6.

Discussion

The results of the present study show that, in the middle and caudal ARC, maximum transcriptional activation of kisspeptin and neurokinin B cells occurs during the LH surge, whereas maximum activation of dynorphin cells is observed 1-2h before the expected onset of sexual behaviour and approximately 6-7 hours before the expected LH surge onset, at a time when plasma oestradiol concentrations are increasing. Furthermore, at this time only, the number of dynorphin cells increased compared to kisspeptin. However, LPS administration in the late follicular phase lowered plasma oestradiol concentrations and prevented dynorphin and kisspeptin cell activation in the middle and caudal ARC. In contrast, insulin prevented dynorphin activation in half the treated animals, whereas kisspeptin activation was stimulated in all insulin-treated animals. Neurokinin B activation was not altered by either treatment. These data implicate kisspeptin, neurokinin B and dynorphin (KNDy cells) as differential mediators of oestradiol feedback at different times within the follicular phase to elicit the GnRH/LH surge, and furthermore, alterations after LPS or insulin were associated with disruption of the LH surge.

Kisspeptin is a potent stimulator of the GnRH neuroendocrine system in sheep (Smith *et al.,* 2008, Lehman *et al.,* 2010a). Cells containing kisspeptin are involved in the oestradiol positive feedback mechanism, which leads to the GnRH/LH surge and ovulation in a large number of species (Clarkson *et al.* 2008, Clarkson and Herbison 2009, Oakley *et al.*

2009, Smith *et al.* 2009, Lehman *et al,* 2010b). However, the time of kisspeptin cell activation relative to the stimulatory oestradiol signal, as well as the location of the kisspeptin cells that respond within the ovine ARC, varies between studies, gonadal state (i.e., OVX *versus* intact) and method of detection (i.e., immunohistochemistry *versus* in situ hybridization). For example, Estrada *et al.,* (2006) report an increase in kisspeptin mRNA in the caudal ARC during the pre-ovulatory period, with further activation in the rostral ARC during oestrus in intact ewes, whereas, Smith *et al.,* (2009) found activated kisspeptin cells in the middle and caudal ARC of OVX ewes 1h after an oestradiol treatment known to stimulate positive feedback. However, the same study (Smith *et al.,* 2009) reports an increase in kisspeptin mRNA, in the middle and caudal ARC, only during the late follicular phase. We have recently mapped kisspeptin cell activation at various times during the follicular phase of intact ewes and found that the percentage of activated kisspeptin cells increased in the rostral, middle and caudal ARC during the LH surge (Chapter 3). A similar finding has been reported by Merkley *et al.,* (2009), with an increase in kisspeptin cell activation in all areas of the ARC in OVX animals undergoing an LH surge. The present study also concurs with our previous findings, as we observed a gradual increase in the percentage of activated kisspeptin cells in the middle and caudal ARC during the follicular phase with a further two-fold increase during an LH surge. Furthermore, during the surge, the percentage of kisspeptin cells that co-expressed dynorphin decreased (i.e., there were more single labelled kisspeptin cells) compared to other times in the follicular phase, suggesting an overall shift towards excitation. In addition, the percentage of activated kisspeptin cells increased as plasma oestradiol values increased, whereas there was an inverse association with decreasing progesterone concentrations.

Neurokinin B is a member of the tachykinin neuropeptide family and is abundant in the ARC of many species (Abel *et al.,* 1999, Goubillion *et al.,* 2000, Latronico *et al.,* 2009). Central administration of a neurokinin B receptor agonist stimulates LH secretion in sheep and monkeys (Billings *et al.,* 2010; Ramaswamy *et al.,* 2010), whereas in humans, mutations of either neurokinin B or its receptor (neurokinin 3 receptor; NK3R) are associated with gonadotrophin deficiency and pubertal failure (Topaloglu *et al.,* 2009). In the present study, the percentage of activated neurokinin B cells gradually increased in

parallel with increasing plasma oestradiol concentrations during the follicular phase with maximum activation during the LH surge. A similar pattern was observed for kisspeptin activation; however, there was no abrupt increase in neurokinin B during the LH surge as observed with kisspeptin. Even though neurokinin B appears to be a stimulating factor of the GnRH/LH surge in the ewe, the mechanism by which this may be achieved is not yet clear as NK3R are not co-localised in GnRH neurones but on KNDy cells (Amstalden *et al.,* 2009). A proposed model for the actions of neurokinin B to control KNDy cell activity is outlined in Lehman *et al.,* (2010b) and Maeda *et al.,* (2010), suggesting that neurokinin B/NK3R signaling plays a role in facilitating and synchronizing activation of kisspeptin neurones. This mode of secretion is proposed for the control of GnRH pulses; however, a similar mode of action could also be employed for the control of the GnRH surge mechanism.

Endogenous opioids peptides (EOPs) play an important role in the inhibition of GnRH secretion (Goodman *et al.,* 1995). Recent evidence indicates that dynorphins located in the POA, anterior hypothalamus and ARC are the primary EOP mediating progesterone negative feedback during the luteal phase (Goodman *et al.,* 2004, Faradori *et al.,* 2005). In the present study during the follicular phase, maximum dynorphin cell activation in the middle and caudal ARC occurred when plasma oestradiol concentrations were increasing and plasma progesterone concentrations were already low, approximately 6-7h before the expected LH surge and the concurrent maximum kisspeptin cell activation. Furthermore, at that specific point, and not at other examined times in the follicular phase, the number of dynorphin cells increased compared to kisspeptin-only cells, whereas the percentage of dynorphin cells that co-expressed kisspeptin was at its lowest (i.e., there were more single-labelled dynorphin cells), suggesting a overall shift towards inhibition. In the present study, the percentage of activated dynorphin cells was not associated with increasing plasma oestradiol concentrations, in contrast to a negative relationship with plasma progesterone values. It is possible that, once progesterone influence decreases below a threshold, there is an increase in the percentage of dynorphin cells that are activated, irrespective of oestradiol concentrations.

Our observations are consistent with a role of KNDy cells in the GnRH surge mechanism. KNDy cells become active in the middle and caudal ARC at a time when plasma oestradiol concentrations are increasing (approximately 6-7h before the expected LH surge onset), and remain activated during the late follicular phase and during the LH surge. However, the balance of neuropeptide expression within this cell population varies throughout the late follicular phase. Initially, there is a shift of the net balance towards inhibition by dynorphin, followed by a swing towards excitation by kisspeptin presumably facilitated by neurokinin B under the influence of increasing oestradiol concentrations. As KNDy cells send projections towards the mPOA (Lehman *et al.,* 2010b) and ME (Amstalden *et al.,* 2005, Smith *et al.,* 2011), these signals may be directly or indirectly transmitted to GnRH cell bodies and/or terminals. The physiological role of an increase in dynorphin cell activation prior to the LH surge is not known. However, these observations are consistent with the hypothesis that a reduction in inhibition (disinhibition) of GnRH secretion by endogenous opioid systems in the hypothalamus is permissive of the preovulatory GnRH/LH surge (Karla, 1993, Walsh and Clarke, 1996, Dobson *et al.,* 2003) and may, therefore, be a part of oestradiol positive feedback mechanism. It appears that KNDy cells in the ARC may contribute to this role. Alternatively, in the OVX ewe, an injection of oestradiol known to elicit positive feedback induces an initial negative feedback effect (Clarke *et al.* 1988, Caraty *et al.,* 1989), which may play a role in preventing premature activation of GnRH neurones, allowing an increase in the releasable pool of GnRH as well as increasing GnRH receptor number (Clarke *et al.* 1988, Walsh and Clarke, 1996, Dobson *et al.* 2003). It may be that increasing plasma oestradiol concentrations in the follicular phase of intact ewes produce a similar effect, reflected by an increase in dynorphin cell activation followed by preovulatory positive feedback (accompanied by kisspeptin and neurokinin B activation). However, as the percentage of activated dynorphin cells increases just before the initiation of sexual behaviour, we cannot rule out the possibility that this increase may be involved in the initiation of signs of sexual behaviour.

In the present study, the number of immunoreactive kisspeptin, dynorphin and neurokinin B cells varied during the follicular phase with maximum cell numbers for all three neuropeptides observed in the late, rather than early, follicular phase.

Furthermore, there were more dynorphin immunoreactive cells compared to kisspeptin-only cells in animals 1-2h before the expected onset of sexual behaviour and approximately 6-7h before the expected surge onset. This may be because each neuropeptide co-localised in KNDy cells is influenced by ovarian steroids in different ways. Indeed, OVX (i.e., the lack of steroid influence) increases neurokinin B and kisspeptin gene expression in the ovine ARC, while oestradiol replacement prevents this increase (Pillon *et al.,* 2003, Smith *et al.,* 2007, Smith *et al.,* 2008). Similar results are also seen when kisspeptin protein is measured by immunohistochemistry (Pompolo *et al.,* 2006, Smith et al., 2007). However, OVX reflects a state where GnRH/LH secretion is elevated, indicating that the kisspeptin system mirrors GnRH secretion (Estrada *et al.,* 2006). In contrast, OVX decreases pre-prodynorphin mRNA in the ARC (Faradori *et al.,* 2004), whereas progesterone treatment did not prevent the effect, raising the possibility that oestradiol stimulates pre-prodynorphin expression in this area (Faradori *et al.,* 2005). Therefore, it is not surprising that endogenous fluctuating steroid concentrations in the present study resulted in different activation patterns in kisspeptin, neurokinin B and dynorphin, even though these neuropeptides are known to co-localise in ARC neurones (Goodman *et al.,* 2007). It would be of great interest to perform *in situ* hybridization for both kisspeptin and dynorphin mRNA to detect possible differences in the timing of maximum increase during the follicular phase of intact ewes to confirm the present findings.

Dynorphin cells have been observed in the magnocellular neurones of the paraventricular nucleus (Goodman *et al.,* 2007) and pre-prodynorphin mRNA in this area increases in response to psychological stress (i.e., isolation) or dehydration in ewes (Matthews *et al.,* 1993), whereas, there is no change after long-term alterations in bodyweight (Iqbal *et al.,* 2003). However, to the best of our knowledge, there are no equivalent data for the actions of stressors on dynorphin cells located in the ARC. In contrast, a few 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.* 2010), short term fasting (Wahab *et al*. 2010) or administration of LPS (Iwasa *et al.* 2008, Kinsey-Jones *et al.* 2009). In the present study, LPS administration in the late follicular phase lowered plasma oestradiol

concentrations and prevented dynorphin and kisspeptin cell activation in the middle and caudal ARC. Interestingly, plasma oestradiol concentrations decreased 8h after the administration of LPS, whereas a decrease in the percentage of activated dynorphin neurones occurred much sooner (3h after treatment). In contrast, the decrease in percentage kisspeptin cell activation was evident later (12h after LPS treatment). It has been suggested that there are at least two mechanisms integrated during LPS disruption of the ovarian cycle; one involving disruption of GnRH/LH pulses thereby reducing oestradiol secretion, and the other preventing the ability of the surge-generating mechanism to respond to the preovulatory increase in oestradiol (Battaglia *et al.,* 1999, Karsch and Battaglia, 2002). Our present data indicate that dynorphin inhibition is involved in the second rather than the first option, as it occurred before the suppression of oestradiol. In contrast, kisspeptin cell activation may have been inhibited either due to the prevention of dynorphin increase (thus, disrupting a necessary chain of events leading to the GnRH surge), or due to decreased GnRH/LH pulsatility and consequent decrease in oestradiol. In this aspect, there are various factors that may act as mediators of this disruption. Cortisol suppresses pulsatile LH secretion in a dose-dependent fashion (Debus *et al.,* 2002). However, in the present study, cortisol increased to maximum concentrations 2h after LPS and is, thus, a potential candidate for the immediate inhibition of dynorphin neurones and, therefore, the surge mechanism. In accordance, Pierce *et al.,* (2009) and Wagenmaker *et al.,* (2009a) report that cortisol disrupts the positive feedback effect of oestradiol to trigger an LH surge. Indeed, ~ 70% of ERα containing cells co-localise glucocorticoid receptors type II (GRII) in the ARC (Dufourny and Skinner, 2002) and it is likely that at least some of these are in KNDy cells, although this remains to be investigated. We observed an increase in progesterone concentrations after LPS, however, the timing of increased values varied considerably between animals, from 2h to 10h after treatment and, therefore, we cannot hypothesise which mechanism might be affected by this increase. It is noteworthy that progesterone has been implicated in both inhibition of GnRH pulses (Karsch *et al.,* 1987) and blocking the surge mechanism (Kasa-Vubu *et al.,* 1992, Skinner *et al.,* 1998, Richter *et al.,* 2002, Smith *et al.,* 2003, Richter *et al.,* 2005). The potential influence of pyrexia remains to be determined; however, our previous studies showed that maximum body temperatures occurred 4h after treatment (Fergani *et al.,* 2011) indicating that pyrexia, presumably via

prostaglandins, may contribute to the attenuation of GnRH pulses. Nonetheless, as these animals did not have an LH surge at the same time as controls, we conclude that the inhibition of dynorphin and kisspeptin cell activation in the middle and caudal ARC is a major contributing factor to LH surge disruption in response to an immune/inflammatory challenge in the ewe. This compliments our recent results in which the absence of an LH surge was accompanied by a failure of ERα-containing neurone activation (Chapter 4).

Interestingly, there was a split response in the insulin-treated animals with two animals showing a decrease in the percentage of dynorphin cells that were activated (similar to LPS; insulin-responders), whereas the remaining two were similar to controls (insulin-non-responders). A similar divergence was observed in a previous study (Fergani *et al.,* 2011) when 10 out of 20 animals treated with insulin did not have a delay in sexual behaviour or the LH surge (insulin-non-delayed subgroup). It is plausible that the animals with no dynorphin inhibition would have gone on to have an LH surge at a similar time as the controls, whereas the others would have had a delayed surge, similar to LPS treated animals. Interestingly, plasma cortisol concentrations increased equally in both insulin sub-groups. This was also observed in our earlier study indicating that cortisol is not solely responsible for the LH surge disruption after insulin (Fergani *et al.,* 2011). From the present results, we can also conclude that cortisol is not the key disruptor of dynorphin activation. In contrast, the percentage of activated kisspeptin neurones increased 3h after treatment in the middle and caudal ARC in both insulin subgroups. This implicates KNDy cells as mediators of metabolic cues to influence reproduction, even though the reason for this differential effect on kisspeptin and dynorphin activation is not understood. Interestingly, neurokinin B activation was not altered by either stressor. The reason for this is not known, however, this finding provides evidence that neurokinin B is not a target for stressors and suggests that it may act as a facilitator in communicating signals between KNDy cells (Lehman *et al.,* 2010b, Meada *et al.,* 2010), but is not sufficient to stimulate an LH surge.

In conclusion, follicular phase ewes, 1-2h before the expected onset of sexual behaviour and approximately 6-7h before the expected LH surge onset, have cells within the ARC that contain ERα and respond to increasing plasma oestradiol concentrations by becoming transcriptionally active (Chapter 4). Furthermore, activation of ERα cells is maintained throughout the late follicular phase and during the LH surge (Chapter 4). The results of the present study provide evidence that this activation pattern may reflect KNDy neurone activation in the middle and caudal ARC and suggests that these cells play a role in the GnRH surge mechanism. However, the balance of neuropeptide expression within this cell population appears to vary throughout the late follicular phase; initially, there is a shift of the net balance towards inhibitory dynorphin followed by a swing towards excitatory kisspeptin presumably facilitated by neurokinin B. By contrast, acute LPS treatment prevented the LH surge from occurring and this was accompanied by a failure of kisspeptin and dynorphin neurone activation. Interestingly, insulin prevented dynorphin activation in two of the treated animals, whereas kisspeptin cell activation was stimulated in all insulin-treated animals, implicating KNDy cells as mediators of metabolic cues to influence reproduction. This indicates that KNDy cell activation is part of a physiological chain of events leading up to the GnRH/LH surge and alteration after insulin or LPS is associated with stress-induced suppression of reproductive parameters. Further studies, using *in situ* hybridisation at various times in the follicular phase to detect potential differences in the activational patterns of dynorphin and kisspeptin, would be beneficial.

Acknowledgements

Thanks are due to Nigel Jones and the farm staff for care of the animals; Hilary Purcell, David Jones and Peter Taylor for technical assistance; and Prof A Parlow and NIAMDD, USA for LH standard preparations. We are also grateful to Richard Morris for his guidance in immunohistochemical techniques, and Dr. Michael Morris for help with animal observations.

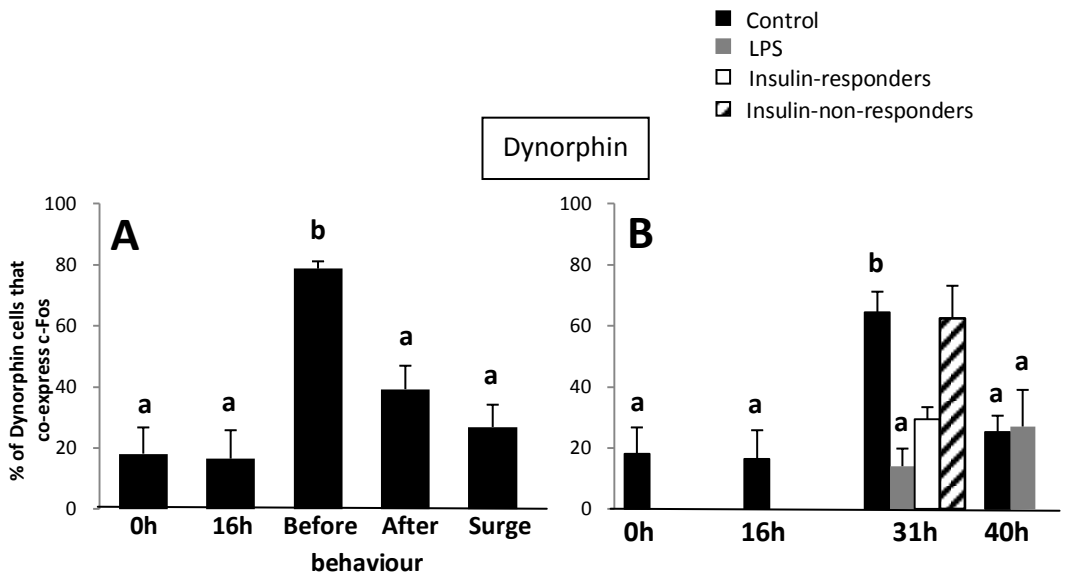

Fig. 1 Mean % (±SEM) dynorphin cells that co-express c-Fos in the middle and caudal ARC at various times during the follicular phase of intact ewes as determined by dual-label immunohistochemistry. Animals in Panel A are grouped according to time as well as hormonal and sexual behavioural status (see text); i.e., grouped into those killed at 0h and 16h after PW (n=5 for both), those killed before the onset of sexual behaviour (Before behaviour, n=3), those killed after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). In Panel B, animals are grouped according to killing 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) and insulin at 31h (insulin-responders, n=2; white bars and insulin-non-responders, n=2; hatched bars). Due to a split response in dynorphin co-expression with c-Fos after insulin treatment, statistical analysis was not carried out but the data are presented for information. Treatment with insulin or LPS was at 28h after PW. Within each panel, differences between the percentages are indicated by different letters at the top of each bar.

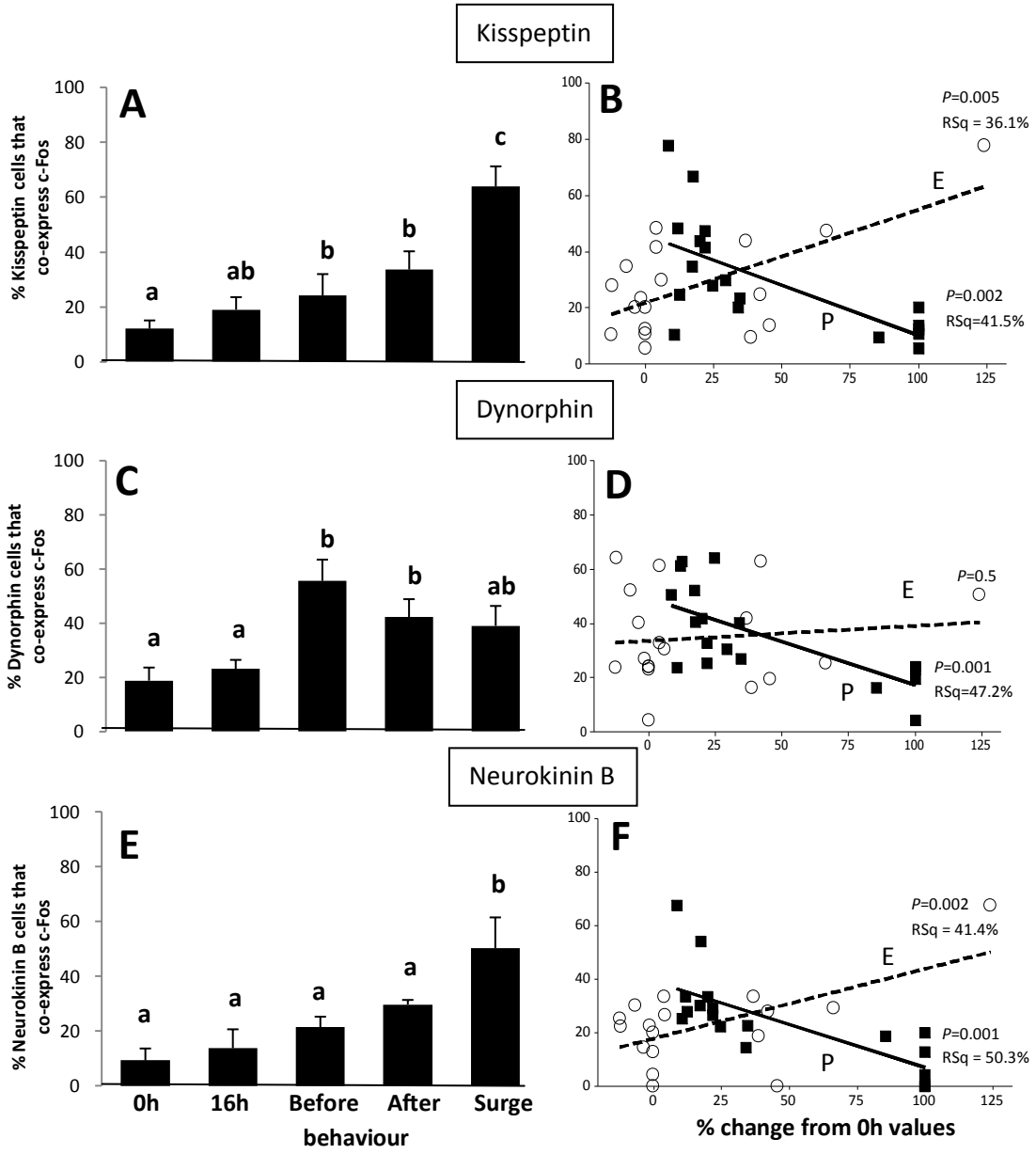

Fig. 2 Mean % (±SEM) kisspeptin (A), dynorphin (C) and neurokinin B (E) containing cells that co-express c-Fos in the middle and caudal ARC at various stages during the follicular phase of control ewes as determined by dual- and triple-label immunohistochemistry (neurokinin B/c-Fos and kisspeptin/dynorphin/c-Fos, 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 ($P<0.05$). Panels B,D,F: Regression graphs showing the association between the % kisspeptin (B), % dynorphin (D) and % neurokinin B (F) cells that co-express c-Fos in the middle and caudal 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.

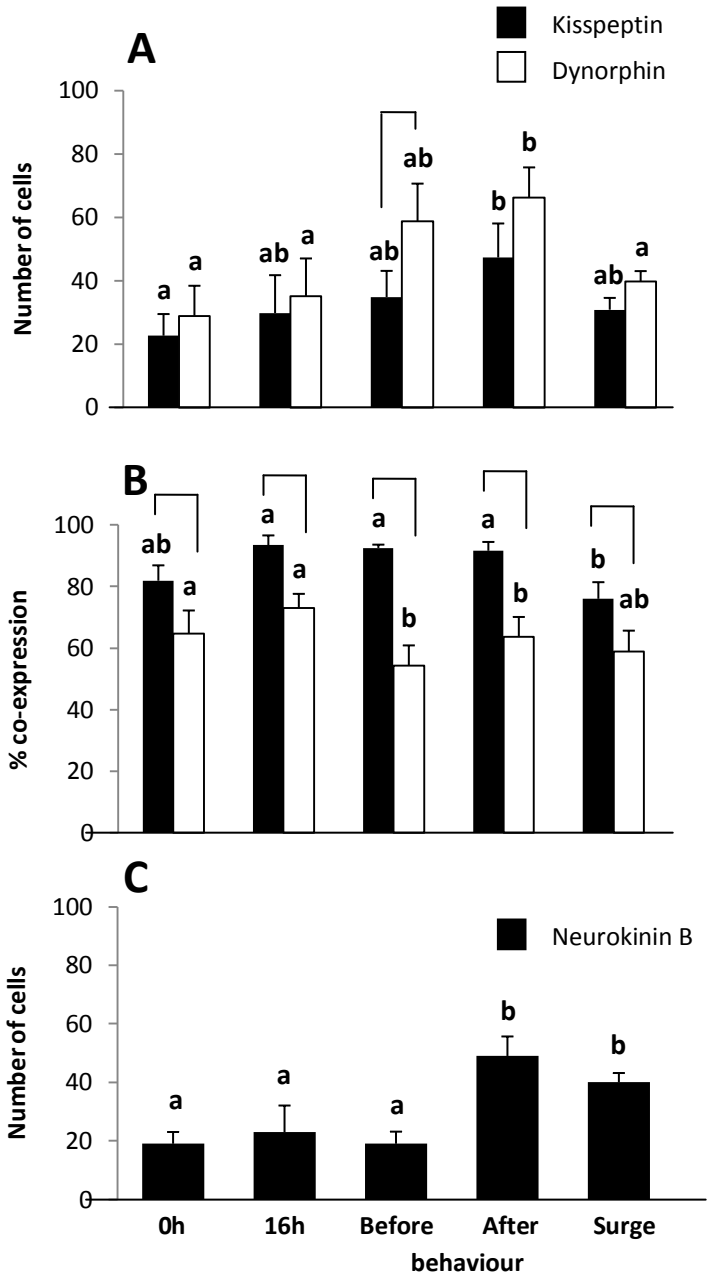

Fig 3. A: Mean (±SEM) number of kisspeptin (black bars) and dynorphin (white bars) cells. B: Mean % (±SEM) kisspeptin cells co-expressing dynorphin (black bars) and dynorphin cells co-expressing kisspeptin (white bars). C*:* Mean (±SEM) number of neurokinin B cells (black bars). Mean (±SEM) numbers and percentages are per section from the middle and caudal ARC at various stages during the follicular phase of control ewes as determined by dual- and triple-label immunohistochemistry (neurokinin B/c-Fos and kisspeptin/dynorphin/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). Differences between cell types are linked with a line (P<0.05).

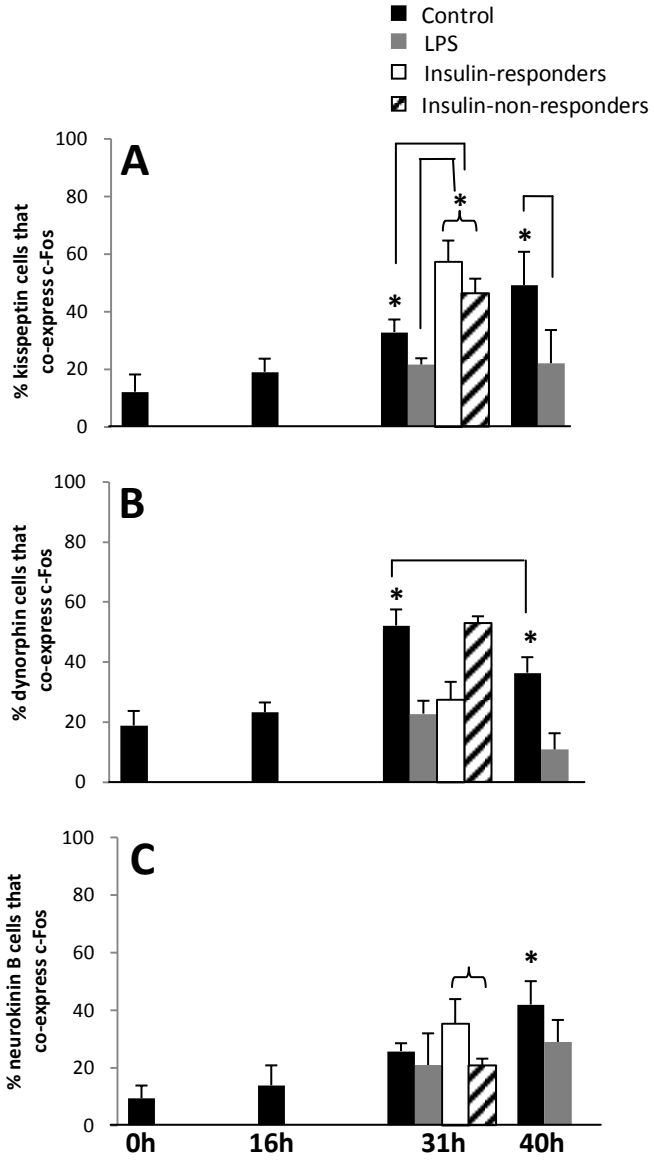

Fig 4. Mean % (±SEM) kisspeptin (A), dynorphin (B) and neurokinin B (C) cells that co-express c-Fos in the middle and caudal ARC at various times during the follicular phase of control and treated ewes as determined by dual- and triple-label immunohistochemistry (neurokinin B/c-Fos and kisspeptin/dynorphin/c-Fos, respectively). Animals are grouped according to killing time after PW i.e., control ewes at 0h, 16h, 31h and 40h (n=4-6 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars) and insulin at 31h (insulin-responders, n=2; white bars and insulin-non-responders, n=2; hatched bars). Due to the split response in dynorphin co-expression with c-Fos after insulin treatment, statistical analysis was not carried out but the data are presented for information. However, there were no split responses observed in kisspeptin and neurokinin B co-expression with c-Fos and therefore statistical analysis was carried out in both groups combined (n=4). Treatment with insulin or LPS was at 28h after PW. Panel A: * P<0.05 compared to 0h and 16 h control groups. Panel B: * P<0.05 compared to all other groups. Panel C: * P<0.05 compared to 0h and 16 h control groups. Differences between groups are also linked with a line (P<0.05).

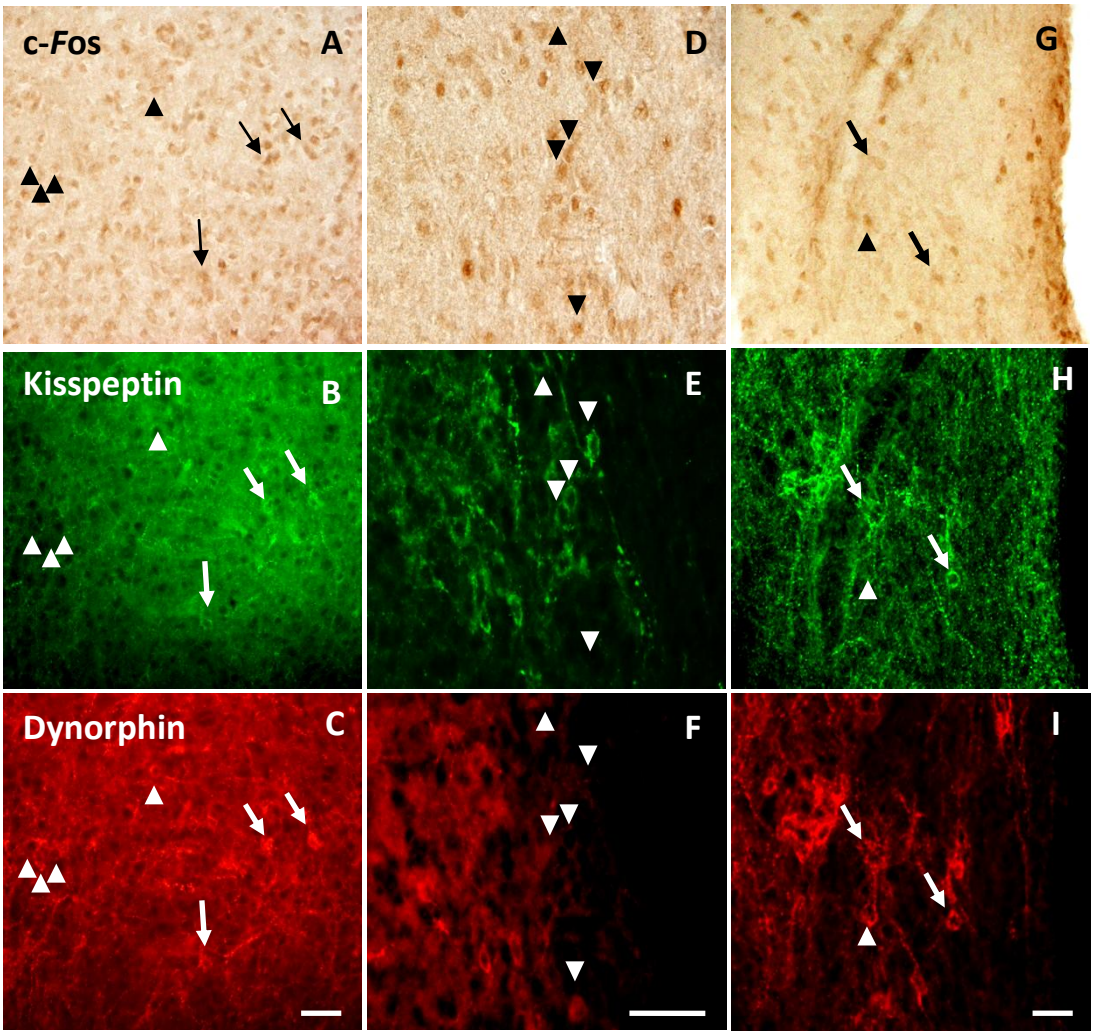

Fig. 5 Sets of photomicrographs in the ARC nucleus that were triple-labelled for c-Fos containing cells (A,D,G) and co-expression with kisspeptin (B,E,H) and dynorphin A (C,F,I) in control animals at 31 h after PW (A,B,C), at 40h after PW (i.e., during the surge; D,E,F) as well as 3h after LPS treatment in the late follicular phase (G,H,I). Examples of dual- and triple-labelled cells are marked with arrowheads and arrows, respectively. Note the presence of dual-labelled cells (arrowheads) reflecting the imbalance in neuropeptide expression in intact ewes during the follicular phase. Original magnification: ×20 (A,B,C,G,H,I), original magnification:×40 (D,E,F).

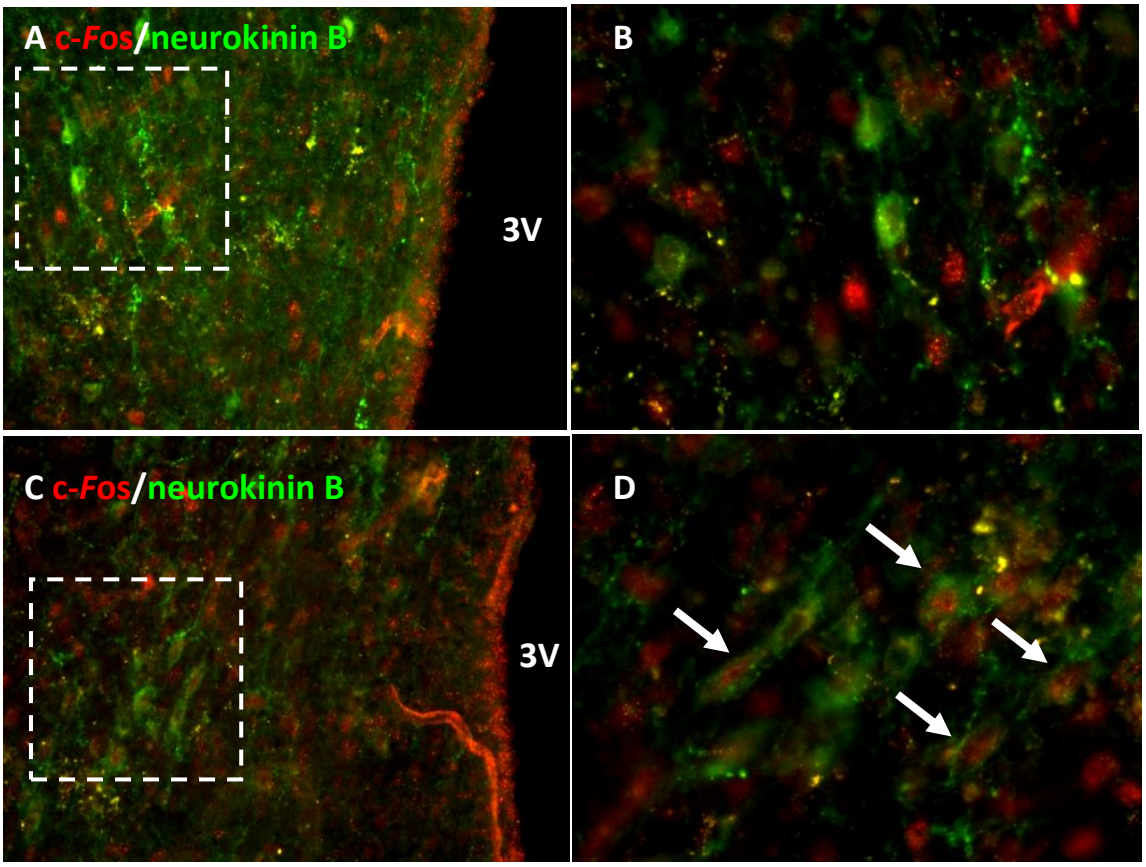

Fig. 6 Photomicrographs from the ARC nucleus that were dual-labelled for neurokinin B cells and their co-expression with c-Fos in control animals at 31h after PW (A and B) as well as during the LH surge (C and D). The *right panels* in each section are the higher magnifications (original magnification: ×40) of the *boxed areas* shown in the *left panels* (original magnification: ×20). *White arrows* indicate examples of dual-labelled cells. 3V = third ventricle.

Table 1. Mean (± SEM) number of cells expressing kisspeptin, dynorphin or neurokinin B immunoreactivity in the middle and caudal ARC as determined by dual- and triple-label immunohistochemistry (neurokinin B/c-Fos and kisspeptin/dynorphin/c-Fos, respectively) in ewes examined at various times after PW.

	Kisspeptin	Dynorphin	Neurokinin B
0 h	22.6 ± 6.8	28.9 ± 9.5	19.1 ± 4,0
16 h	29.8 ± 12.1	35.1 ± 11.9	23.3 ± 9.1
31 h control	37.2 ± 3.0*	59.9 ± 6.2*#	30.0 ± 7.5
31h LPS	29.0 ± 7.4	28.9 ± 5.4	28.8 ± 10.3
31h insulin-responders	25.2 ± 9.0	15.0 ± 6.0	17.7 ± 1.2
31h insulin-non-responders	26.0 ± 2.2	30.7 ± 1.2	34.5 ± 8.0
40h control	42.1 ± 11.9*	53.5 ± 11.8**	39.2 ± 6.9
40 h LPS	33.6 ± 9.5	35.6 ± 9.1	29.2 ± 2.3

Kisspeptin: * $P<0.05$ within a column, greater than 0h control group.

Dynorphin: * $P<0.05$ within a column, greater than 0h, 16h and LPS groups.

** $P<0.05$ within a column, greater than 0h.

# $P<0.001$ within a row, greater than kisspeptin and neurokinin B.

Table 2. Mean (± SEM) percentage of kisspeptin cells that co-express dynorphin and mean (± SEM) percentage of dynorphin cells that co-express kisspeptin in the middle and caudal ARC as determined by triple-label immunohistochemistry (kisspeptin/dynorphin/c-Fos) in ewes examined at various times after PW.

	% kisspeptin cells that co-express dynorphin	% dynorphin cells that co-express kisspeptin
0h	81.9 ± 10.5#	64.7 ± 7.4
16h	93.4 ± 3.2*#	73.1 ± 4.5*
31h control	90.1 ± 1.8*#	56.8 ± 4.9
31h LPS	70.3 ± 9.6	69.0 ± 9.8
31h insulin-responders	76.0 ± 7.3	67.1 ± 9.8
31h insulin-non-responders	97.3 ± 2.7	51.9 ± 11.5
40h control	83.8 ± 5.8#	63.6 ± 5.8
40h LPS	84.5 ± 4.5	75.6 ± 2.8*

% kisspeptin cells that co-express dynorphin:

*P<0.05 within a column, greater than 31h LPS.

# P<0.05 within a line, greater than % dynorphin cells that co-express kisspeptin.

% dynorphin cells that co-express kisspeptin:

*P<0.05 greater than all other groups.

Chapter 6

Chapter 6

Dopamine, β-endorphin, neuropeptide Y and somatostatin co-expression with c-Fos in the mediobasal hypothalamus at different times during the follicular phase of intact ewes, and alteration after insulin or endotoxin.

Keywords: Dopamine, β-endorphin, neuropeptide Y, somatostatin, c-Fos, ewe, insulin, LPS, stress, GnRH surge, LH surge.

Abstract

The aim of the present study was to determine the activation pattern of dopamine, β-endorphin, neuropeptide Y and somatostatin cells in the mediobasal hypothalamus (MBH) during the follicular phase of intact ewes, as well as investigating whether these patterns are altered by stressors. Follicular phases of intact ewes were synchronised with progesterone vaginal pessaries. Control animals were killed at 0, 16, 31 and 40h (n=5-6 per group) after progesterone withdrawal (PW; time zero). At 28h, groups of animals received insulin (4 iu/kg) or endotoxin (LPS; 100 ng/kg) and were subsequently killed at 31h (insulin; n=5 and LPS; n=5) or 40h (LPS; n=5). LH surges occurred only in 40h control ewes. Activation patterns were established using c-Fos (a marker for neuronal activation) with dual-label immunofluorescence. In the ARC of control ewes, the percentage of dopamine cells co-expressing c-Fos (i.e., activated cells) were decreased before the onset of sexual behaviour and remained low until the LH surge (from 70 to 26%; $P<0.05$) whereas β-endorphin activation was lower during the LH surge (from 41 to 10%; $P<0.05$). By contrast, neuropeptide Y activation tended to be higher during the surge (from 21 to 36%; $P<0.08$) whereas somatostatin activation in the ARC and VMN was greater during the LH surge compared to other stages in the follicular phase (from 14 and 9% to 47 and 73%, respectively; $P<0.05$). However, insulin increased the percentage of activated β-endorphin, neuropeptide Y and somatostatin cells in the ARC, at 31h after PW (i.e., 3h after treatment; to 71, 72 and 63%, respectively, $P<0.05$ for each comparison, compared to controls killed at the same time), but there was no effect on dopamine activation. Activation of the above neuro-phenotypes in the ARC was not affected by LPS treatment. In the VMN, at 31h after PW (i.e., 3h after insulin or LPS treatment) activation of somatostatin cells was greater in all LPS treated animals (from 8

to 27%; $P<0.05$) but only in two of the insulin-treated animals (to 55 and 76%; insulin-responders) but not in the other two (to 5 and 6%; insulin-non-responders). These results indicate that there is a specific temporal pattern of dopamine, β-endorphin, neuropeptide Y and somatostatin activation in the ARC and VMN during the follicular phase of intact ewes. This is disturbed by acute LPS or insulin administration in the late follicular phase in a stress-specific manner.

Introduction

Gonadotrophin-releasing hormone (GnRH) neurones constitute the final common pathway of a complex neuronal network responding to the circulating steroid hormone milieu to control ovulation and sexual behaviour (Karsch *et al.,* 1997, Caraty *et al.,* 2002). However, the precise mechanisms that control each function are not yet fully understood. In the late follicular phase, increasing concentrations of oestradiol, secreted by the dominant follicle(s), trigger the onsets of the GnRH/LH (luteinising hormone) surges as well as sexual behaviour (de Greef *et al.,* 1987, Moenter *et al.,* 1990, Fabre-Nys and Martin 1991, Xia *et al.,* 1992). However, steroid hormone signals do not impinge directly on GnRH cells as they do not possess progesterone receptors (PR) or oestradiol receptors subtype α (ERα; Shivers *et al*., 1983, Herbison and Theodosis 1992, Lehman and Karsch, 1993, Sullivan *et al.* 1995, Scott *et al.* 2000, Skinner *et al*., 2001). GnRH neurones have been reported to contain oestrogen receptor β (ERβ; Skynner et al., 1999; Hrabovszky et al., 2001, Herbison *et al.* 2001, Jansen *et al.,* 2001, Skinner and Dufourny 2005) but their role is not fully elucidated, although considered minor (Scott *et al.* 2000, Orikasa and Sakuma 2003, Maeda *et al.,* 2010). Thus, it is generally accepted that cells in the brain that possess ERα are the main mediators of the feedback effects of steroid hormones and they in turn secrete neurotransmitters to influence the GnRH network (Caraty *et al*. 1998, Herbison 1998, Smith and Jennes 2001, Herbison 2008).

Various types of stressors have the ability to block or delay the LH surge and sexual behaviour (Dobson *et al.,* 1999, Dobson and Smith 2000, Fergani *et al.,* 2011). We have recently shown that a sudden activation of the hypothalamo-pituitary-adrenal axis in the late follicular phase by the immunological stressor endotoxin (i.e., lipopolysaccharide;

LPS) lowered plasma oestradiol concentrations and delayed the onsets of pre-copulatory behaviours, oestrus and the LH surge of free-running intact ewes; whereas the metabolic stressor insulin-induced hypoglycaemia had the same effect in only 50% of animals (Fergani *et al.,* 2011). Immunohistochemical analysis of c-Fos protein expression (a marker of neuronal transcription activation; Hoffman *et al.,* 1993) revealed that this disruption is accompanied with the activation of unknown cell types located in the ARC and VMN (Chapter 3).

In the ewe, the mediobasal hypothalamus (MBH; comprising ARC and VMN), contains a dense population of neurones expressing ERα (Blache *et al.,* 1994), and constitutes the major site of action for oestradiol to regulate the induction of both the preovulatory GnRH surge and sexual behaviour (Blache *et al.,* 1991, Caraty *et al.,* 1998). These brain areas also contain several types of neurones, sub-populations of which co-express ERα, including those that contain tyrosine hydroxylase (TH; a biosynthetic enzyme marker for dopamine), β-endorphin and neuropeptide Y in the ARC, as well as somatostatin in the ARC and VMN (Antonopoulos *et al.,* 1989, Lehman *et al.,* 1988, Willoughby *et al.,* 1995). These neuro-phenotypes are therefore, potential candidates to be involved in the generation of the GnRH surge or sexual behaviour. In contrast, stress-induced alterations may play a role in reproductive dysfunction.

During the follicular phase, dopaminergic input to the MBH facilitates the expression of sexual behaviour. Extra-cellular dopamine concentrations are high when peripheral oestradiol values are low, but dopamine concentrations decrease after oestradiol reaches maximum values in the late follicular phase (Melis and Argiolas, 1995, Fabre Nys *et al.,* 2003). It is possible that stress-induced disruption of sexual behaviour may involve extended dopamine accumulation in the MBH. A sub-population of DA neurones in the ARC contain ERα (3-15%; Lehman and Karsch, 1993, Skinner and Herbison, 1997), whereas 20% of dopamine cells provide input towards the VMN (Qi *et al.,* 2008). Therefore, such cells are good candidates to facilitate stimulatory or inhibitory changes.

Opioids are considered as a brake, which must be removed for the LH surge to occur (Kalra, 1993, Dobson *et al.,* 2003). We have recently shown that changes in dynorphin expression are associated with GnRH inhibition during the late follicular phase (Chapter

5), whereas β-endorphin could be acting synergistically in this respect. This latter opioid has also been implicated in the stress-induced disruption of the LH surge after insulin (Dobson and Smith, 2000, Ghuman *et al.,* 2011) but whether this is a stress-specific response remains to be investigated.

In the rat, neuropeptide Y (NPY) is involved in the regulation of GnRH secretion (Kalra *et al.,* 1995, Pelletier *et al.,* 1990, Watanobe and Takebe, 1992, Sahu *et al.,* 1995) but specific actions (inhibitory or stimulatory) depend on the underlying endocrine status (Kalra *et al.,* 1991). However, in sheep, its role is less clear and results are conflicting. Studies using various metabolic stressors implicate NPY neurone activation under conditions of negative energy balance, leading to an increase in NPY mRNA expression (Adam *et al.* 1997, Henry *et al.* 2000). A similar response may be obtained after insulin or LPS administration in the late follicular phase of sheep.

Short-term oestradiol treatment in ovariectomised (OVX) ewes increases somatostatin activation in the VMN in a sex-specific manner (Robinson *et al.,* 2010). Interestingly, in rats, somatostatin is one of the most potent inhibitors of electrical excitability of GnRH neurones identified thus far (Bhattarai *et al.,* 2010) and inhibits the LH surge when administered centrally (Van Vugt *et al.* 2003). It is, therefore, possible that somatostatin containing cells are activated via an unknown mechanism to mediate stress-induced disruption of the LH surge.

In the present study, we examined brain tissue of intact ewes sacrificed at various times during the follicular phase with or without the administration of insulin or LPS. Our objective was to map the pattern of transcriptional activation of dopamine, β-endorphin, NPY neurones in the ARC as well as somatostatin neurones in the ARC and the VMN (by measuring co-expression with c-Fos, a marker of neuronal activation; Hoffman *et al.,* 1993). We also aimed to correlate these patterns with peripheral plasma progesterone and oestradiol concentrations, as well as the exhibition of sexual behaviour and the LH surge. Furthermore, we sought to determine whether the disruption of the surge mechanism after LPS or insulin administration in the late follicular phase involves alteration in the activation of these neuro-phenotypes.

Materials and Methods

Animals, study design and blood sampling procedure

In this investigation we used tissue collected during the breeding season for an earlier study, details of which are given in Chapter 3.

Visual observation of oestrous behaviour

Details are given in Chapter 3.

Tissue collection

Details are given in Chapter 3.

c-Fos and DA or β-endorphin or neuropeptide Y or somatostatin dual-label immunofluorescence

Dual-label immunofluorescence was carried out on 40μm sections containing middle and caudal levels of the ARC (three sections for each level and each neuropeptide combination). 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) in 0.1M phosphate buffer saline, pH 7.2 (PBS). Free-floating sections were washed thoroughly in PBS for 2h to remove the cryoprotectant solution followed by 1h incubation in blocking solution (10% donkey serum in PBS). This was followed by 72h incubation at 4^0C with polyclonal rabbit anti-c-Fos antibody (AB-5, PC38, Calbiochem, Cambridge, MA, USA) at a dilution of 1:5,000. After incubation with the primary antiserum, sections were washed thoroughly and incubated for 2h with donkey anti-rabbit Cy3 (711-165-152, Jackson Immunoresearch, West Grove, PA) diluted 1:500. A second immunofluorescence procedure was then performed, as described above, to localise the second primary antibody: mouse anti-tyrosine hydroxylase serum (MAB318, Millipore, Billerica, MA), at a dilution of 1:20,000 or rabbit-anti-β-endorphin serum (T-4041, Peninsula Laboratories, San Carlos, CA, at a dilution of 1:500), or rabbit-anti-neuropeptide Y serum (N9528, Sigma-Aldrich, UK at a dilution of 1:5,000) or anti-rabbit somatostatin-14 serum (T-4103, Peninsula Laboratories, San Carlos, CA, at a dilution of 1:500), each incubated for 72h at 4^0C and then visualised using donkey-anti-mouse

Dylight 488 (715-485-151, Jackson Immunoresearch West Grove, PA) or donkey-anti-rabbit Dylight 488 (715-485-152, Jackson Immunoresearch West Grove, PA), accordingly, both at a dilution of 1:500. Thereafter, sections were washed with PBS followed by a final wash with double-distilled water, mounted on chrome alum gelatine-coated slides and cover-slipped with Vectashield anti-fading mounting medium (H-1000; Vector Laboratories Ltd, UK). The c-Fos (Ghuman *et al.* 2010), tyrosine hydroxylase (Robinson *et al.,* 2010), β-endorphin (Ghuman *et al.,* 2010), neuropeptide Y (Skinner and Herbison, 1997) and somatostatin-14 (Robinson *et al.,* 2010) antibodies have been validated previously for use in ovine neural tissue. In addition, negative controls omitting one of the primary antibodies completely eliminated the appropriate fluorescence without obviously affecting the intensity of the other fluorescent probe.

Data analysis

Dual-labeled sections were examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1) and photographed by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) using a 20× objective. Photographs acquired with an image analysis program AxioVision (Zeiss Imaging Systems) and consisted of single c-Fos staining, single dopamine or β-endorphin or neuropeptide Y or somatostatin staining as well as a merged image to produce a spectral combination of green (fluorescein) and red (rhodamine). The areas examined were (as defined by Welento *et al.,* 1969): the VMN (4 photographs per section) and the ARC (3 photographs per section; middle and caudal levels). Sections were evaluated unilaterally and each photograph was taken from a random field within each area/section. All photographs were imported into Image J version 1.42q, 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-labelled cells was summed from the photographs of each section and then averaged for each ewe and compared between groups with GLM ANOVA, followed, when appropriate, by Tukey's multiple comparisons *post hoc* test. Mean (±SEM), as presented in figures and results, was calculated by averaging values for each group. For all analyses, statistical significance was considered when $P<0.05$ and tendencies for significance when $0.05>P<0.09$.

The data were analysed in two ways: the first consisted of control ewe data grouped according to time as well as hormonal and sexual behavioural status; i.e., grouped into those killed at 0h and 16h after PW, those killed at 31h after PW but before the onset of sexual behaviour (Before behaviour, n=3), those killed at 31h or 40h after PW, after the onset of sexual behaviour but before exhibiting an LH surge (After behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). This grouping allowed for a detailed comparison of neuropeptide profiles in control animals at different stages of the follicular phase in our intact-ewe model. Secondly, control and treated animals data were grouped according to time of killing after PW, and this was used to compare treatment effects.

During data analysis, it became clear that there was a split response in the insulin group regarding the percentage somatostatin cells that co-expressed c-Fos in the VMN. Therefore this group was separated into two subgroups referred to hereafter as insulin-responders or insulin-non-responders (verified previously in Chapter 3 as with or without c-Fos activation in the paraventricular nucleus, respectively). As this division reduced the group size to n=2/group, statistical analysis was not undertaken, but the data are presented for information. When responses were not different between sub-groups, data were combined and analysed with n=4.

Peripheral plasma progesterone and oestradiol values fluctuated between animals, thus, regression analysis was used to examine the association between the percentage of change from 0h to the two mean consecutive lowest or highest progesterone or oestradiol values, respectively, and the percentage of dopamine, β-endorphin, NPY or somatostatin cells that co-expressed c-Fos (i.e., were activated) in the ARC and VMN of control animals.

Results

Luteinising hormone (LH) and behavioural profiles.

As shown in chapter 3

Control ewes: Dopamine and c-Fos co-expression in the middle and caudal ARC at different stages during the follicular phase, in animals grouped by behaviour.

The percentage of dopamine cells that co-expressed c-Fos decreased in the 'Before behaviour', 'After behaviour' and 'Surge' stages, compared to 0h and 16h (P<0.05 for each comparison; Fig. 1A).

Control ewes: β-endorphin and c-Fos co-expression in the middle and caudal ARC at different stages during the follicular phase, in animals grouped by behaviour.

The percentage of β-endorphin cells that co-expressed c-Fos was greater in the 16h and 'After behaviour' groups compared to 0h and 'Surge' stages (P<0.05 for both comparisons; Fig. 1B).

Control ewes: NPY and c-Fos co-expression in the middle and caudal ARC at different stages during the follicular phase in animals grouped by behaviour.

The percentage of NPY cells that co-expressed c-Fos did not vary during the follicular phase, although, percentages tended to be higher in the 'Surge' group (P<0.08; Fig. 1C).

Control ewes: Somatostatin and c-Fos co-expression in the middle and caudal ARC and VMN at different stages during the follicular phase in animals grouped by behaviour.

The percentage of somatostatin cells that co-expressed c-Fos in the middle and caudal ARC and the VMN was greatest in the 'Surge' group compared to other stages in the follicular phase (P<0.05 for both; Fig. 1D and Fig. 4A). Photomicrographs from the ARC dual-labelled with dopamine or β-endorphin or neuropeptide Y or somatostatin and c-Fos are shown in Fig. 2.

Oestradiol

Oestradiol data have been presented in Chapter 3. Using regression analysis, the percentages of NPY cells that co-expressed c-Fos in the ARC and somatostatin cells that co-expressed c-Fos in the ARC and VMN were positively associated with the percentage change in concentration from 0h to the mean two consecutive highest plasma oestradiol values (P=0.008, RSq=29%, P=0.000, RSq=69% and P=0.000, RSq=78%, respectively; Fig.

3C, 3D and Fig. 4C, respectively) but not for dopamine or β-endorphin cells (P=0.4 and P=0.09; Fig. 3A, 3B).

Progesterone

Progesterone data have been presented in Chapter 3. Using regression analysis, the percentage of dopamine cells that co-expressed c-Fos is positively associated with the percentage change in concentration from 0h to the mean two consecutive lowest plasma progesterone values in the ARC (*P*=0.000 RSq=73%; Fig. 3A) but not for β-endorphin, NPY in the ARC and somatostatin in the ARC and VMN (P=0.6, P=0.2, P=0.1 and P=0.1, respectively; Fig. 3B, 3C, 3D and Fig. 4C).

Cortisol

Cortisol data have been presented in Chapter 3.

Control, insulin or LPS: Dopamine and c-Fos co-expression in the middle and caudal ARC at different times during the follicular phase, in animals grouped by hours after PW.

The percentage of dopamine cells that co-expressed c-Fos n controls was greatest at 0h and 16h control groups compared to other times examined in the follicular phase (*P*<0.05 for both; Fig. 5A). LPS or insulin had no effect on the percentage of dopamine cells that co-expressed c-Fos at any time examined after treatment (Fig. 5A). The number of dopamine immunoreactive cells was increased in the 40h control compared to 0h controls (*P*<0.05; Table 1).

*Control, insulin or LPS: β-endorphin and c-*Fos *co-expression in the middle and caudal ARC at different times during the follicular phase, in animals grouped by hours after PW.*

The percentage of β-endorphin cells that co-expressed c-Fos in controls was greater in the 16h and 31h control groups than the 0h and 40h control groups (*P*<0.05 for both; Fig. 5B). However, at 31h after PW (i.e., 3h after insulin administration), the percentage of β-endorphin cells that co-expressed c-Fos increased in both insulin sub-groups compared to other groups (from 38.3 ± 4.8 to 70.9 ± 1.7%; combined insulin-responders and insulin-non-responders; *P*<0.05; Fig. 5B). LPS did not have the same effect (Fig. 5B). The number of β-endorphin immunoreactive cells was greater in the 31h and 40h

control groups and 31h and 40h LPS groups (compared to 0h controls $P<0.05$ for all; Table 1).

Control, insulin or LPS: NPY and c-Fos co-expression in the middle and caudal ARC at different times during the follicular phase, in animals grouped by hours after PW.

The percentage of NPY cells that co-expressed c-Fos in controls did not vary during the follicular phase of control ewes (Fig. 5C). However, at 31h after PW (i.e., 3h after insulin administration) the percentage of NPY cells that co-expressed c-Fos increased in both insulin sub-groups (from 18.9 ± 2.0 to 72.2 ± 5.5%; combined insulin-responders and insulin-non-responders; $P<0.05$; Fig. 5C). LPS did not have an effect (Fig. 5C); furthermore, the number of NPY immunoreactive cells did not vary across the follicular phase of controls and was not affected by treatment (Table 1).

Control, insulin or LPS: Somatostatin and c-Fos co-expression in the middle and caudal ARC and VMN at different times in the follicular phase, in animals grouped by hours after PW.

The percentages of somatostatin cells that co-express c-Fos in the middle and caudal ARC and in the VMN were increased at 40h compared to other times examined in the follicular phase ($P<0.05$; Fig. 5D and Fig. 4B, respectively). However, at 31h after PW (i.e., 3h after insulin administration) the percentage of somatostatin cells that co-expressed c-Fos in the middle and caudal ARC increased in both insulin sub-groups (from 14.3 ± 3.3% to 63.0 ± 9.4%; combined insulin-responders and insulin-non-responders; $P<0.05$; Fig. 5D). LPS did not have an effect and results were not different to controls at any time (Fig. 5D). By contrast, at 31h after PW (i.e., 3h after LPS or insulin administration), the percentage of somatostatin cells that co-expressed c-Fos in the VMN increased in the LPS group ($P<0.05$) and the insulin-responders sub-group; Fig. 4B). At 40h after PW (i.e., 12h after LPS administration), when the majority of animals were having an LH surge, LPS and control groups were not different (Fig. 4B). The number of somatostatin immunoreactive cells in the middle and caudal ARC, as well as in the VMN, did not vary during the follicular phase of control and was not affected by treatment (Table 1). Photomicrographs from the VMN dual-labelled with somatostatin and c-Fos are shown in Fig. 6.

Discussion

The aim of the present study was to map the activation pattern of various neuropeptides implicated in the sequence of events leading up to the GnRH/LH surge and sexual behaviour in intact ewes and determine how these altered after the application of two stressors; insulin or LPS. We focused on two brain areas that are important for the steroid feedback control of GnRH release in the sheep: the middle and caudal ARC and the VMN (Blache *et al.,* 1991, Caraty *et al.,* 1998). Within these regions, we investigated neurones that contain dopamine, β-endorphin, NPY and somatostatin.

Pattern of dopamine activation in the middle and caudal ARC during the follicular phase of control ewes.

There are several populations of dopaminergic cell groups within the hypothalamus (Tillet and Thibault, 1989) and some of these have been implicated in the control of reproduction. For example, DA neurones located in the A15 retrochiasmatic area are indirectly stimulated by oestradiol (as they do not contain ERα) to inhibit GnRH secretion during anoestrus but not the breeding season (Bertrand *et al.,* 1998, Anderson *et al.,* 2001, Adams *et al.,* 2006, Goodman *et al.,* 2010). During an induced follicular phase in OVX ewes, dopamine was implicated in the control of sexual behaviour (Fabre-Nys and Gelez, 2007). In the present study, the percentage of activated dopamine neurones in the middle and caudal ARC was greater in the early follicular phase (0h and 16h after PW, i.e., 12-28h before the expected onset of sexual behaviour). Thereafter, there was a decrease and dopamine neurone activation was low in animals before and during sexual behaviour and the LH surge. These results are consistent with a biphasic role of dopamine as described by Fabre-Nys et al., (1994, 2003). Using microdialysis, Fabre-Nys *et al.,* (1994) showed that in progesterone-primed OVX ewes, extra-cellular concentrations of dopamine in the MBH are high at the end of the luteal phase. This is followed by a sharp decrease after oestradiol administration and preceding the onset of sexual behaviour (Fabre-Nys *et al.,* 1994). Our own data indicate that the activational patterns of dopamine cells are highly co-related with plasma progesterone concentrations. It is, therefore, probable that progesterone, via dopamine, acts as a brake on the neuronal circuits mediating the expression of sexual behaviour. Thereafter,

increasing oestradiol concentrations remove dopamine inhibition and sexual behaviour is initiated 12h later. The present findings suggest that the source of dopaminergic input to the MBH is derived, at least in part, from cells located in the middle and caudal ARC. We have recently shown that ERα-containing cells in the VMN are activated concurrently with the exhibition of sexual behaviours providing evidence that the VMN may be involved in this function (Chapter 4). Indeed, 20% of dopamine neurones provide input towards the VMN (Qi *et al.,* 2008) providing a possible pathway for the control of female sexual behaviour. However, the cells that receive dopaminergic input within the VMN remain to be elucidated.

Pattern of dopamine activation in the middle and caudal ARC during the follicular phase of LPS or insulin treated ewes.

In the present study, sexual behaviour did not occur within the study time-frame in the majority of treated ewes, whereas in a few ewes, oestrous signs commenced but were disturbed by the stressors. Progesterone and dopamine administration facilitate the expression of sexual behaviour while oestradiol concentrations are low but are inhibitory once oestradiol values increase above a threshold (Fabre-Nys *et al.,* 2003). This may constitute a potential pathway for the stress-induced suppression of oestrous behaviour observed in the present study. However, LPS or insulin did not alter dopamine activation in the ARC and, therefore, it remains to be established whether other dopamine populations, such as those in the PVN, may account for the disruption.

In the present study, cortisol increased to maximum concentrations immediately after the administration of LPS or insulin (i.e., 2h after treatment) and this steroid is, therefore, a potential candidate for the inhibition of sexual behaviours (Pierce *et al.,* 2008). However, cortisol is able to inhibit oestradiol-induced sexual receptivity (i.e., oestrus) but not other components of sexual behaviour which were also disturbed in our study (Pierce *et al.,* 2008, Papargiris *et al.,* 2011). Therefore, cortisol may contribute, but cannot be the sole mediator of stress-induced disruption of sexual behaviour. We also observed a very small increase in plasma progesterone concentrations after LPS. This subtle change may be one of the mechanisms by which all sexual behaviours were blocked (Scaramuzzi et al., 1971, Fabre-Nys 1998, Fabre-Nys and Gelez 2007).

Pattern of β-endorphin activation in the middle and caudal ARC during the follicular phase of control ewes.

β-endorphin cells represent another potential relay point for conveying the influence of oestradiol onto GnRH neurones. In the middle and caudal ARC, 15-20% of β-endorphin cells contain ERα (Lehman and Karsch, 1993) whereas, β-endorphin or pro-opiomelanocortin (POMC) fibres directly innervate GnRH cells in the rat (Leranth *et al.,* 1988) and monkey (Thind and Goldsmith*,* 1988). In the ewe, β-endorphin neurones project from the ARC directly to the POA (Whisnant *et al.,* 1992, Jansen *et al.,* 2003, Dufourny *et al.* 2005) where the majority of GnRH cells are located (Lehman *et al.* 1986), although direct contact has not been confirmed. In the present study, activation of β-endorphin cells increased 16h after PW, remained in this state throughout the late follicular phase but decreased in animals exhibiting an LH surge. These results are consistent with those of Domanski *et al.* (1991), who demonstrated a decrease in β-endorphin concentrations in the ARC/ME (median eminence) of intact ewes 10–12 h before the onset of the preovulatory GnRH and LH surges. Similar results have been obtained in rats (Wise *et al.,* 1990, Bohler *et al.,* 1991, Rosie *et al.,* 1992, Petersen *et al.,* 1993). We have recently shown that dynorphin cell activation in the middle and caudal ARC also increases prior to, but not during, the LH surge (Chapter 5). These observations are consistent with the hypothesis that a reduction in inhibition (disinhibition) of GnRH secretion by endogenous opioid systems in the hypothalamus is permissive of the preovulatory GnRH/LH surge (Kalra, 1993, Walsh and Clarke, 1996, Dobson *et al.,* 2003). Increased opioid influence during the follicular phase may play a role in preventing premature activation of GnRH neurones, allowing an increase in the releasable pool of GnRH, as well as increasing GnRH receptor number (Clarke *et al.* 1988, Walsh and Clarke, 1996, Dobson *et al.* 2003).

Our results conflict with those of Walsh *et al.* (1998), who did not observe any difference in POMC mRNA levels in the ARC of ewes between the luteal phase of the cycle compared to the follicular phase (24h after cloprostenol) and the period of the preovulatory LH surge. Taylor *et al.,* (2007) 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 methods of

detection (i.e., immunohistochemistry of c-Fos induction in β-endorphin cells versus POMC gene transcription). Indeed, 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).

Pattern of β-endorphin activation in the middle and caudal ARC during the follicular phase of LPS or insulin treated ewes.

Insulin treatment increased the percentage of activated β-endorphin neurones 3h after treatment in the middle and caudal ARC in both insulin subgroups. Indeed, opioids have been implicated in LH suppression after insulin treatment in the ewe (Clarke *et al.,* 1990, Smith *et al.,* 2003). Furthermore, infusion of the non-specific opioid antagonist, naloxone just before insulin administration prevented the delay in the onset of the LH surge observed after insulin alone (Dobson and Smith, 2000). Our results conflict with those of Ghuman *et al.* (2011), who observed a decrease in β-endorphin activation in the ARC after insulin treatment. However, the dose used in that study was larger than the one used here (5 iu/kg *versus* 4 iu/kg) and was administered later in the follicular phase (34h after PW versus 28h after PW); this could account for the divergent results. Indeed, this leads us to believe that the dose of insulin and the level of hypoglycaemia, as well as the time of administration may be of importance in disruptive mechanisms. LPS did not have the same effect, and β-endorphin activation was not altered. Therefore, it appears that β-endorphin is a mediator of insulin-induced disruption of the LH surge, but this effect is stressor specific.

Pattern of NPY activation in the middle and caudal ARC during the follicular phase of control ewes.

NPY is widely distributed in the central nervous system but its role in reproductive functions is attributed to the population of neurones located in the ARC (Kalra and Crowley, 1992). In the rat, NPY is involved in the regulation of GnRH secretion (Kalra *et al.,* 1991, Pelletier *et al.,* 1992, Watanobe and Takebe, 1992, Sahu *et al.,* 1995) but specific actions depend on the endocrine status. For example, in rats, NPY stimulates GnRH release in the presence of oestradiol, but inhibits GnRH release during oestradiol

absence (Kalra *et al.,* 1992). In the sheep, the role of NPY in the regulation of GnRH is less clear. NPY administered intracerebroventricularly (icv) suppressed release of LH in OVX and OVX oestradiol-treated sheep, whereas NPY mRNA was more abundant during the luteal phase than prior to the surge (Malven *et al.,* 1992, McShane *et al.,* 1992, Estrada *et al.,* 2003). However, in follicular phase ewes, icv administration of anti-NPY serum delayed the onset of the preovulatory LH surge, implying a stimulatory role in this process (Porter *et al.,* 1993). Furthermore, icv NPY infusion stimulated the synthesis and storage of LH but not its release in prepubertal lambs (Wankowska *et al.,* 2002, Wójcik-Gładysz *et al.,* 2003). In addition, a stimulatory effect on GnRH release by NPY infusion into the ME was observed in intact sexually active ewes, but only in the follicular, and not in the luteal, phase of the oestrous cycle (Advis *et al.,* 2003). In the present study, NPY activation did not vary across the follicular phase, however, it tended to be higher in animals that were exhibiting an LH surge. Furthermore, this pattern of activation was positively correlated to plasma oestradiol concentrations. The reason for this divergence between results in not known, however, studies in rats (Crowley and Kalra, 1987), rabbits (Khorram *et al.,* 1987), and monkeys (Woller and Terasawa, 1992) provide evidence for the existence of two mechanisms, one inhibitory and one stimulatory, through which endogenous NPY regulates GnRH. About one third of NPY neurones co-localise γ-aminobutyric acid (GABA; Horvath *et al.,* 1997). Furthermore, all GnRH neurons express GABA receptors in rats (Clarkson and Herbison, 2006) and GABA terminals are in close contact with GnRH neurones in the mPOA of sheep (Jansen *et al.,* 2003, Sliwowska *et al.,* 2006,). Interestingly, GABA release can hyperpolarise or depolarise GnRH neurones depending on the presence of oestradiol (DeFazio *et al.,* 2002, Moenter and DeFazio, 2005, Ghuman *et al.,* 2008) in agreement with opposing NPY effects on LH secretion. However, the origin of the GABAergic inputs to GnRH neurones is not fully elucidated, and therefore further studies are necessary to explore NPY/GABA interaction modulating GnRH secretion.

Pattern of NPY activation in the middle and caudal ARC during the follicular phase of LPS or insulin treated ewes.

NPY neurones have been considered good candidates to operate as neuroendocrine mediators, linking alterations in energy balance signals to the reproductive axis (Hill *et*

al., 2008). According to this paradigm, NPY neurones are activated under conditions of negative energy balance, leading to an increase in NPY mRNA expression (Adam *et al.* 1997, Henry *et al.* 2000). In accordance, our data show that insulin treatment in the late follicular phase, increased NPY neurone activation 3h after treatment in the middle and caudal ARC in both insulin subgroups. Indeed, repeated administration of insulin up-regulates NPY mRNA in the hypothalamus of rats (Briski *et al*., 2010), whereas, secretory activity of NPY cells is enhanced by starvation (Chailou *et al.,* 2002) or long-term under-nutrition (Polkowska and Gładysz, 2001) in sheep. Furthermore, we report that LPS does not have the same effect and NPY neurone activation is not altered after the application of this stressor in the late follicular phase.

Pattern of somatostatin activation in the middle and caudal ARC and VMN during the follicular phase of control ewes.

Within the MBH of the ewe, numerous somatostatin neurones are present in the ARC and the ventrolateral division of the VMN (Willoughby *et al.,* 1995, Bruneau and Tillet, 1998). However, the physiological role of these cells is not fully understood. In the present study, there was an increase in the percentage of activated somatostatin neurones in the ARC and the VMN during the LH surge compared to other stages in the follicular phase. Furthermore, this activational pattern was positively correlated to plasma oestradiol concentrations. In the ovine VMN, 30% somatostatin neurones express ERα (Herbison, 1995, Scanlan *et al.,* 2003), and this accounts for 70% of the total number of ERα-containing cells in the VMN (Herbison, 1995). Projections from the VMN terminate in close opposition to about half of the GnRH cells bodies in the ovine mPOA (Goubillon *et al.,* 2002) and somatostatin receptor 2 (SSTR2) is co-expressed within ovine GnRH neurones (Robinson *et al.,* 2010). Our results indicate that somatostatin neurones in the VMN are directly regulated by oestradiol to transmit positive feedback to GnRH neurones during the surge. We also provide evidence for a similar effect of oestradiol in somatostatinergic neurones of the ARC. However, as relatively fewer cells in this population co-express ERα (13%; Scanlan *et al.,* 2003), the variation in ARC somatostatin activation observed in the present study could also result from an indirect action of oestradiol via inter-neurones. Indeed, oestradiol treatment influences somatostatin

neurones in the VMN and ARC of guinea pigs and rats (Baldino *et al.,* 1988, Werner *et al.,* 1988, Zorrilla *et al.,* 1990, Dufourny and Warembourg, 1999).

In accordance with the present data, in OVX ewes injected intramuscularly with oestradiol to induce preovulatory GnRH and LH surges, the percentage of activated somatostatin neurones in the VMN and ARC was higher during the surge compared to non-oestradiol treated ewes (Scanlan *et al.,* 2003). However, Robinson *et al.,* (2010) demonstrated that a short (6h) treatment with oestradiol increased activation in somatostatin containing neurones in the VMN, and this was sex-specific. Pillon *et al.,* (2004) reported a similar finding, with increased preprosomatostatin (PPS) mRNA expression in the VMN and the ARC after 4h of oestradiol administration. The latter two studies, in similar ewe models, suggest that somatostatin is activated in the early stages of the surge induction process. The reason for this time difference in somatostatin activation between the latter two studies and the present one is not known. It is possible that somatostatin neurones activated in the early stages of surge generation are the 30% of somatostatin neurones that contain ERα, while those activated at the time of the GnRH surge belong to the 70% of non-ERα somatostatin cells. Interestingly, icv administration of somatostatin abolished LH pulsatility and dramatically decreased the mean basal level of LH secretion (Pillon *et al.,* 2004). This is most likely achieved via GnRH neurone inhibition, as somatostatin inhibits *in vitro* release of GnRH from rat mediobasal hypothalamic slices (Rotsztejn *et al.* 2002). Together, all these observations lead to a hypothesis that the majority of somatostatin neurones are activated during the surge and this may be important for termination of GnRH/LH secretion.

We cannot rule out that the alterations in somatostatin activation that we observed could be related to the regulation of growth hormone (GH) secretion. Oestradiol modulates GH release particularly at the time of the preovulatory LH surge, when a coincident surge of GH has been observed in sheep (Landefeld and Suttie, 1989, Malven *et al.,* 1995, Dutour *et al.,* 1997, Scanlan and Skinner, 2002).

Pattern of somatostatin activation in the middle and caudal ARC and VMN during the follicular phase of LPS or insulin treated ewes.

Our data support a role for somatostatin in stress-induced disruption of the LH surge. We observed an increase in somatostatin activation in the VMN, 3h after LPS treatment in all the animals and two insulin-treated animals (insulin-responders), but not the other two. By contrast, ARC somatostatin neurones were activated in both insulin sub-groups. There are several hypotheses for the role of somatostatin during stress. First, as mentioned above, somatostatin is one of the most potent inhibitors of electrical excitability of GnRH neurones identified thus far in rats (Rotsztejn *et al.* 2002, Bhattarai *et al.,* 2010) and inhibits the LH surge when administered centrally (Van Vugt *et al.* 2003). It is, therefore, possible that somatostatin cells are activated via an unknown mechanism to mediate stress-induced disruption of the LH surge via direct action on GnRH cells (Robinson *et al.,* 2010). Second, in the rat, either acute or chronic stressful stimuli suppress pulsatile secretion of GH from the pituitary gland (Lenox *et al.* 1979) and this suppression is mediated by hypothalamic somatostatin (Terry *et al.* 1976). Specifically, it has been postulated that nutrient deficiency (such as fasting) as well as central administration of interleukin-1 (a cytokine product of LPS administration) inhibits GH secretion in the sheep by a mechanism dependent on the stimulation of hypothalamic somatostatin (Rettori *et al.,* 1994, Polkowska, 1996, Henry *et al.,* 2001, Córdoba-Chacón *et al.,* 2011). In the rat, at least 70% of somatostatin neurones from the periventricular region, but not those in MBH, project to the ME and form a final common pathway for the regulation of GH secretion from the anterior pituitary (Kawano and Daikoku 1988, Merchenthaler *et al.* 1989). Somatostatin neurones in the MBH could be involved in this mechanism indirectly, via the activation of periventricular somatostatin neurones. However, the mechanism by which these cells are activated remains unknown. It is unlikely that cortisol mediates this effect as the VMN contains very few glucocorticoid receptor type 2 (Dufourny and Skinner, 2002).

Interestingly, we observed a split response in the insulin-treated animals with two showing an intense activation of somatostatin cells in the VMN (similar to LPS), whereas, the other two were similar to controls. An equivalent divergence was observed in our previous studies when 10 out of 20 animals treated with insulin had a delay in sexual behaviour and the LH surge, whereas the other half did not (Fergani *et al.,* 2011). Furthermore, the same two animals used in the present study had no activation in

somatostatin cells and did not display an intense transcriptional activation in the paraventricular nucleus (PVN) or VMN (insulin-non-responders; Chapter 3). Taken together, these observations indicate that somatostatin activation in the VMN may be an important factor in the stress-induced disruption of the LH surge. Further studies with a larger number of animals should confirm this finding.

In conclusion, our results indicate that the activational patterns of dopamine, β-endorphin, NPY and somatostatin in the middle and caudal ARC and in the VMN differ during the follicular phase of intact ewes. Specifically, events leading up to the GnRH/LH surge include: activation of dopamine cells decreasing 12h before the expected onset of sexual behaviour as well as an activational decrease in β-endorphin and increase in NPY and somatostatin cells during the GnRH/LH surge. However, this activational pattern was disturbed by acute stressors in the late follicular phase. Interestingly, insulin-treatment increased β-endorphin, NPY and somatostatin cell activation in the ARC, which was evident 3h after treatment, whereas LPS had no effect, indicating that these changes are stressor-specific. By contrast, somatostatin activation increased in the VMN 3h after LPS treatment in all animals and two insulin-treated animals (insulin-responders), but not the other two, indicating that this neuropeptide may be important in the stress-induced disruption of the LH surge.

Acknowledgments

Thanks are due to Nigel Jones and the farm staff for care of the animals; Hilary Purcell, David Jones and Peter Taylor for technical assistance; and Prof A Parlow and NIAMDD, USA for LH standard preparations. We are also grateful to Dr. Richard Morris for his guidance in immunohistochemical techniques as well as Dr. Michael Morris for help with animal observations.

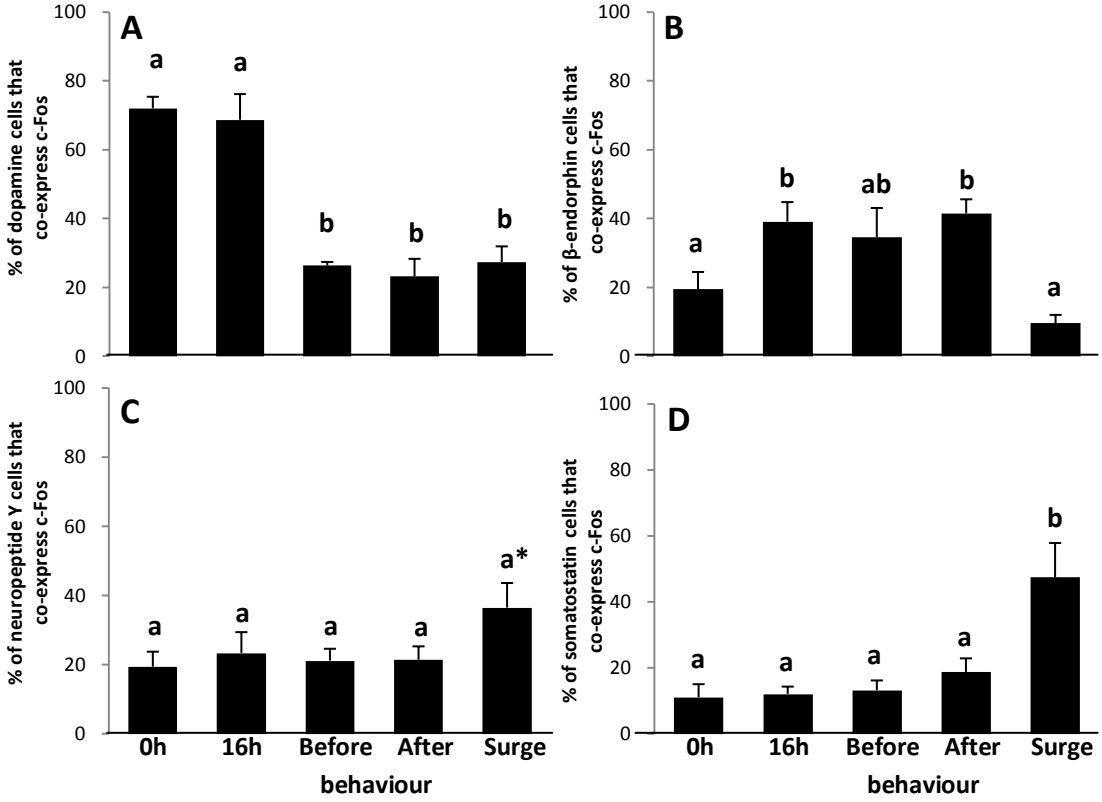

Fig. 1 Mean % (±SEM) dopamine (A), β-endorphin (B), neuropeptide Y (C) and somatostatin (D) cells that co-express c-Fos in the middle and caudal ARC at different stages during the follicular phase of control ewes as determined by dual-immunofluorescence. Animals are grouped according to time as well as hormonal and behavioural status; i.e., grouped into those killed at 0h and 16h after PW (n=4-5), those killed before the onset of sexual behaviour (before behaviour, n=3), those killed after the onset of sexual behaviour but before exhibiting an LH surge (after behaviour, n=5) and those killed after the onset of both sexual behaviour and during the 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). * P<0.08.

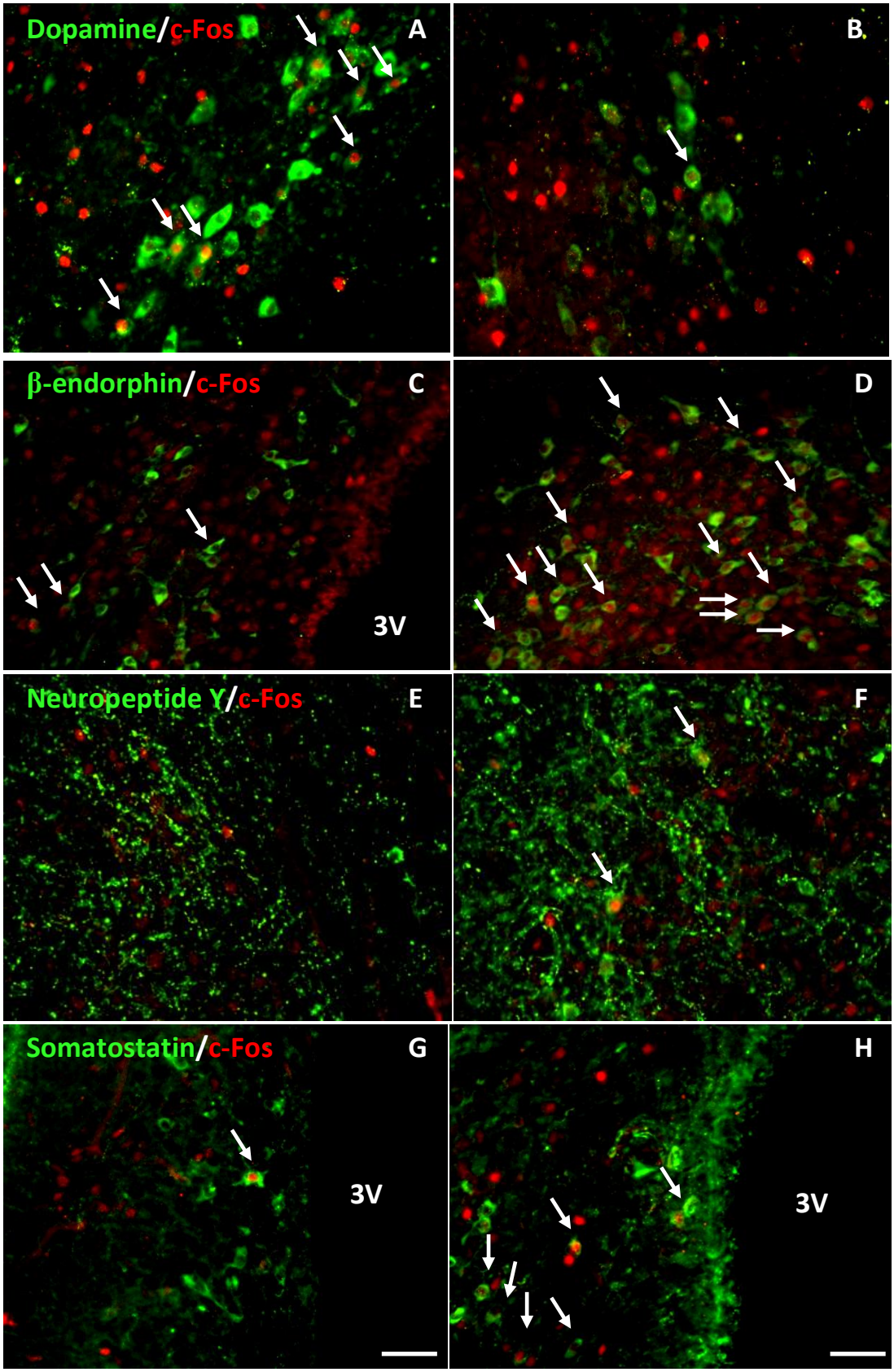

Fig. 2 Photomicrographs from the ARC nucleus that were dual-labelled with c-Fos and tyrosine hydroxylase (a marker for dopamine; A,B) or β-endorphin (C,D), or neuropeptide Y (E,F) or somatostatin (G,H) in 0h control (A), 31h control (E,G), 40h control (during the LH surge; B,C,H) or 31h insulin-responders (D,F). *White arrows* indicate examples of dual-labelled cells. 3V = third ventricle. (Scale bar: 50 μm).

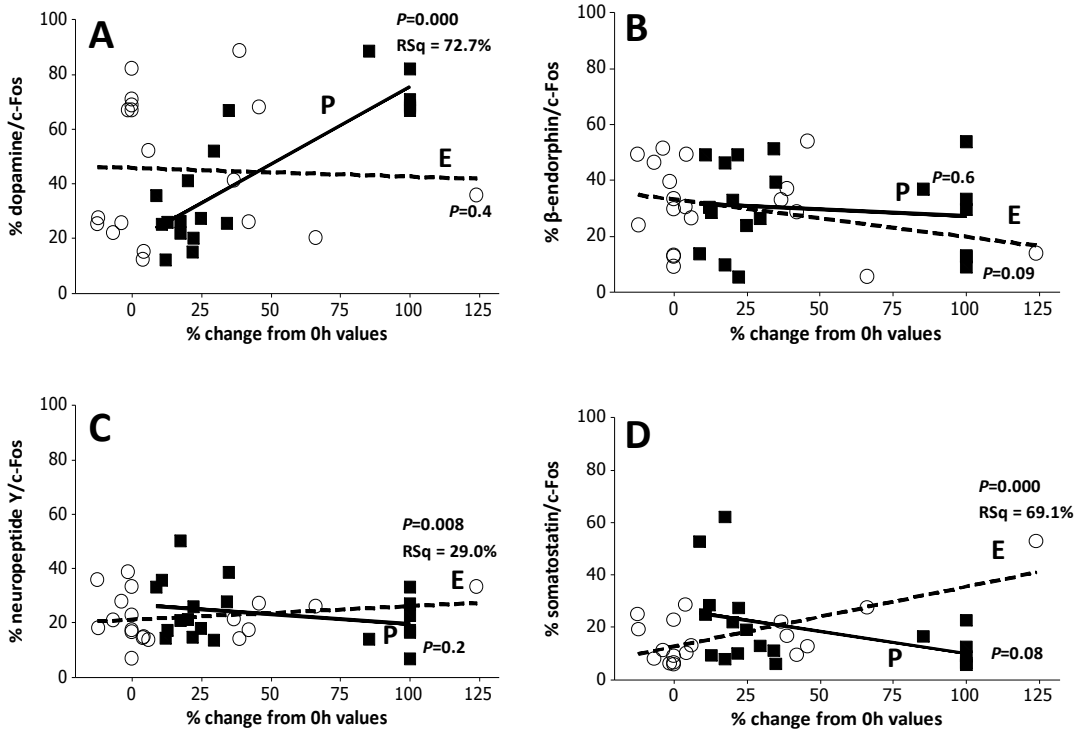

Fig. 3. Regression graphs showing the association between the % dopamine (A; % dopamine/c-Fos), % β-endorphin (B; % β-endorphin/c-Fos), % neuropeptide Y (C; % neuropeptide Y/c-Fos) and % somatostatin (D; % somatostatin/c-Fos) cells that co-express c-Fos in the middle and caudal ARC of control ewes against 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.

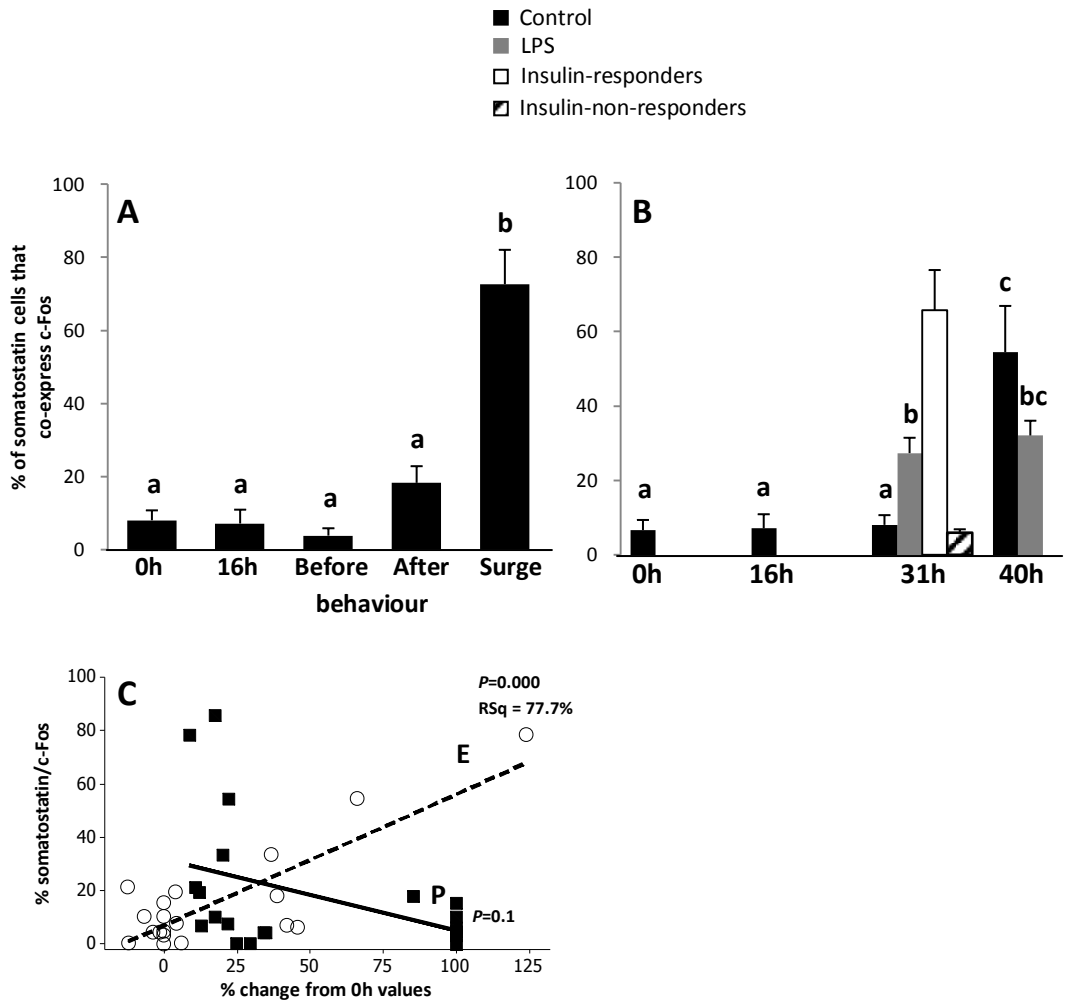

Fig 4. Mean % (±SEM) somatostatin cells that co-express c-Fos in the VMN during the follicular phase of control and treated ewes as determined by dual-immunofluorescence. Animals in Panel A are grouped according to time as well as hormonal and behavioural status; i.e., grouped into those killed at 0h and 16h after PW (n=4-5), those killed before the onset of sexual behaviour (before behaviour, n=3), those killed after the onset of sexual behaviour but before exhibiting an LH surge (after behaviour, n=5) and those killed after the onset of both sexual behaviour and during the LH surge (Surge, n=3). In Panel B animals are grouped according to killing time after PW i.e., control ewes at 0h, 16h, 31h and 40h (n=4-6 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars) and insulin at 31h (insulin-responders, n=2; white bars and insulin-non-responders, n=2; hatched bars). Due to the split response in insulin-treated animals, statistical analysis was not carried out and the data are presented for information. Treatment with insulin or LPS was at 28h after PW. Within each panel, differences between the percentages are indicated by different letters on top of each bar (P<0.05). Panel C: Regression graph showing the association between the % somatostatin cells that co-express c-Fos in the VMN (% somatostatin/c-Fos) of control ewes 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.

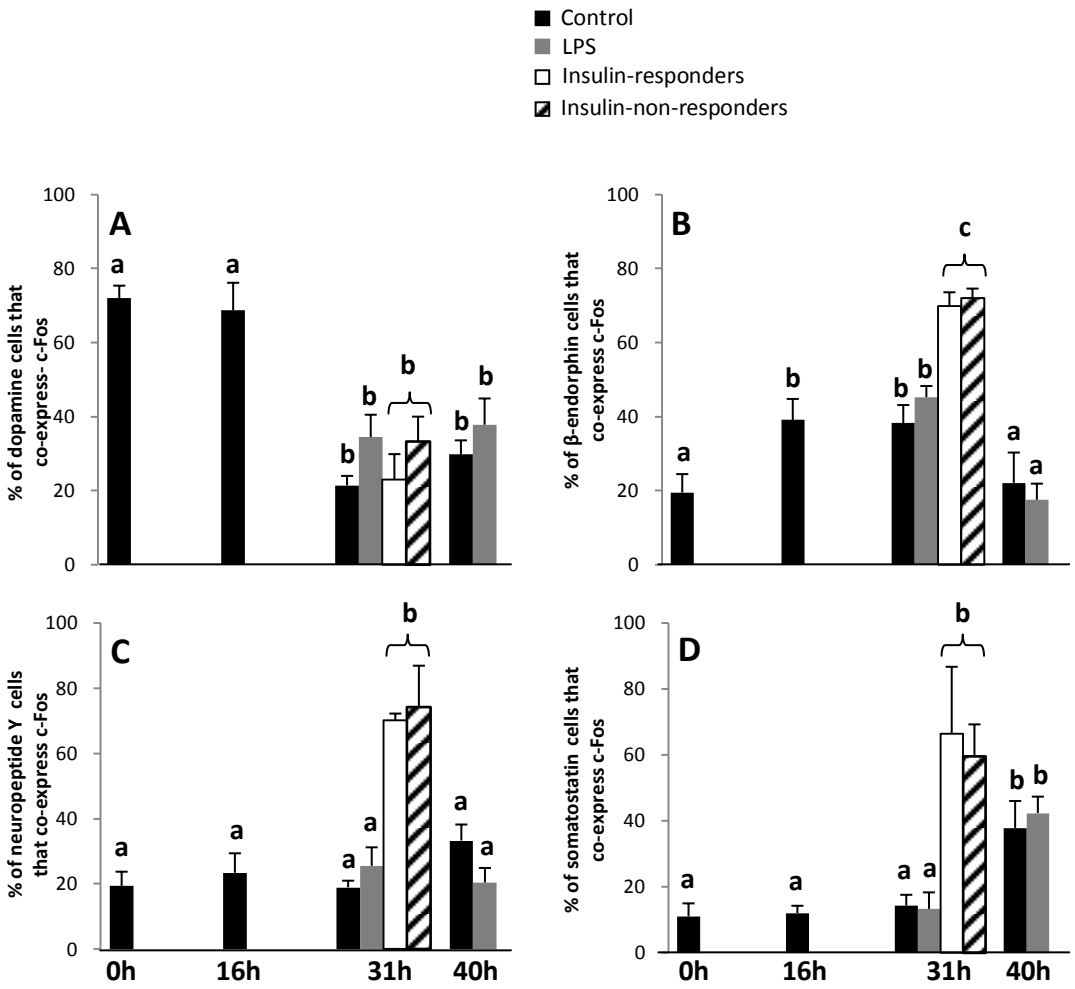

Fig 5. Mean % (±SEM) dopamine (A), β-endorphin (B), neuropeptide Y (C) and somatostatin (D) cells that co-express c-Fos in the middle and caudal ARC across the follicular phase of control ewes as determined by dual-immunofluorescence. Animals are grouped according to killing time after PW i.e., control ewes at 0h, 16h, 31h and 40h (n=4-6 per group; black bars) as well as after LPS at 31h and 40h (n=4 for both times; grey bars). There were no split responses observed in the 31h insulin-treated animals i.e., insulin-responders (n=2; white bars) and insulin-non-responders (n=2; hatched bars) and therefore statistical analysis was carried out in both groups combined (n=4). Treatment with insulin or LPS was at 28h after PW. Within each panel, differences between the percentages are indicated by different letters on top of each bar (P<0.05).

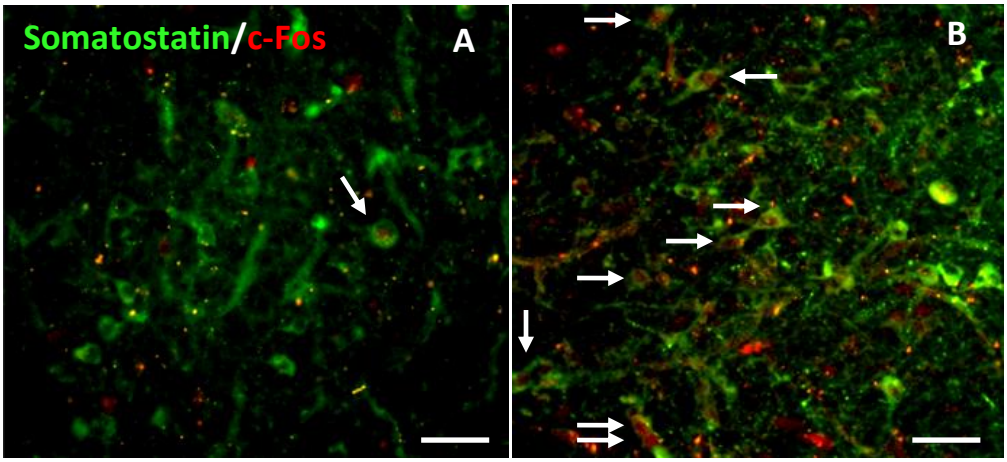

Fig. 6 Photomicrographs from the VMN that were dual-labelled for somatostatin cells and their co-expression with c-Fos. In 31h control (A) and LPS treated (B) ewes. *White arrows* indicate examples of dual-labelled cells. 3V = third ventricle. (Scale bar: 50 μm).

Table 1. Mean (±SEM) number of cells expressing dopamine, β-endorphin, neuropeptide Y or somatostatin immunoreactivity in the middle and caudal ARC and VMN as determined by dual-immunofluorescence in ewes examined at various times after PW.

	Dopamine (ARC)	β-endorphin (ARC)	Neuropeptide Y (ARC)	Somatostatin (ARC)	Somatostatin (VMN)
0 h	28.0 ± 6.2	47.5 ± 11.3	22.6 ± 9.1	48.9 ± 15.1	29.8 ± 9.9
16 h	42.3 ± 10.5	56.5 ± 15.4	38.2 ± 9.5	55.6 ± 16.9	25.9 ± 15.9
31 h control	40.3 ± 15.5	83.3 ± 13.0*	22.6 ± 3.6	36.5 ± 10.3	22.3 ± 6.0
31h LPS	39.5 ± 14.8	109.8 ± 19.5*	26.4 ± 7.6	32.1 ± 5.6	28.0 ± 5.7
31h insulin-responders	75.5 ± 9.5	71.8 ± 6.2	21.3 ± 12.8	36.0 ± 4.8	54.8 ± 14.8
31h insulin-non-responders	70.0 ± 5.2	81.8 ± 20.7	32.3 ± 13.8	29.2 ± 9.8	44.5 ± 19.5
40h control	60.5 ± 4.5*	92.0 ± 9.4*	33.8 ± 6.1	58.1 ± 16.0	36.5 ± 17.2
40 h LPS	53.1 ± 10.8	93.9 ± 6.6*	41.4 ± 17.6	52.0 ± 21.1	58.8 ± 18.0

Dopamine: * $P<0.05$ within a column, greater than 0h control group.

β-endorphin: * $P<0.05$ within a column, greater than 0h control group.

Chapter 7

Conclusions and Further work

The studies contained in this thesis have contributed significantly to answering the following questions:

How does the application of acute stressors in the late follicular phase affect the timing of sexual behaviour and the LH surge?

One of the aims in the present thesis was to compare the effects of two different stressors insulin or LPS, administered during the late follicular phase (i.e., 28h after PW and ~ 8-9h before the expected LH surge onset), on sexual behaviour and LH profiles. Control animals in both of the studies began exhibiting sexual behaviour approximately 28-30h after PW and had an LH surge ~ 36-38h after PW, but these were delayed by the application of stressors. In the first study (Chapter 2), LPS delayed both the onsets of behaviour and the LH surge by ~22 h. By contrast, responses to the administration of insulin resulted in two sub-groups: the timing of pre-ovulatory behaviour and the LH surge was not affected in half the ewes (10 out of 20; insulin-non-delayed) but, in the other half (10 out of 20; insulin-delayed), the onset of behaviour was delayed by ~7 h and the LH surge by ~17 h, resulting in a separation of the two events by 10h. Similarly, in our second study (Chapter 3, 4, 5, 6), LPS treated animals did not have an LH surge at the same time as controls, and sexual behaviour did not occur within the study time-frame for the majority of treated ewes, whereas in a few ewes, oestrous signs commenced but were disturbed by the stressor.

The fact that insulin-treated animals were able to overcome the inhibition of the behaviour generating mechanism quicker than the inhibition of the GnRH surge generating mechanism by 10 h, suggests that these two events are regulated independently and provides a model that can be used to identify the specific neuronal systems that control behaviour distinct from those initiating the GnRH surge. In the present thesis, it was not possible to make this comparison, as brain tissue was retrieved from insulin-treated animals at a time when the distinction between delayed and non-delayed animals could not be made. The separate mechanisms might be elucidated by

repeating the study with a larger number of animals and more time points for brain sampling.

As successful reproduction requires follicular maturation and oestradiol biosynthesis, induction of the LH surge, ovulation, and expression of sexual behaviour to be co-ordinated within a tight time-frame, the separation observed after a 'weaker' stressor such as insulin may have detrimental effects on fertility via mechanisms impacting on oocyte quality and embryo viability. The effects of insulin on these parameters remain to be investigated.

The reason for the 10h disparity between sexual behaviour and LH surge onsets observed after insulin-treatment is not known, however, it is possible that GnRH pulses were restored before the pituitary regained sensitivity to GnRH and, therefore, behaviour was driven centrally by the GnRH pulses (Caraty *et al.,* 1998, Caraty *et al.,* 2002). Simultaneous GnRH/LH pulse and oestrus monitoring, although practically challenging, could help to elucidate this point.

How does the application of acute stressors in the late follicular phase effect peripheral hormone concentrations?

Oestradiol

In both studies, plasma oestradiol concentrations in control animals were elevated by ~28h, reached a maximum at ~32h-36h after PW and then began to decrease just as the LH surge occurred. In study 1, oestradiol concentrations decreased after either stressor, and remained low for a period equivalent to the LH surge delay. It is, therefore, hypothesised that when a stressor is applied during the late follicular phase, the duration of the LH surge delay is related to the duration of oestradiol signal disruption. Interestingly, Insulin-non-delayed ewes had two types of oestradiol profiles, with some ewes appearing to be unaffected by treatment, whilst others had a sudden decrease in concentrations followed by a quick recovery. In study 2, oestradiol concentrations decreased within 2h after insulin treatment, but only after 8h following LPS

administration. The reason for this time lag in oestradiol decrease between the two stressors is not known, however, this finding suggests that the mechanisms of GnRH/LH pulse inhibition after the two stressors may be different.

Progesterone

In control animals, progesterone concentrations were decreased by 16h after PW and remained low during the follicular phase. In study 1, insulin-delayed and LPS groups had an increase in progesterone concentrations and this subtle change may have been one of the mechanisms by which all sexual behaviours and the LH surge were blocked in these groups. In addition, an increase in progesterone (probably of adrenal gland origin) was the only stress-hormone difference between insulin-delayed and insulin-non-delayed sub-groups, indicating that this steroid may play an important role in stress-induced disruption of reproductive parameters.

Cortisol

Cortisol increased to maximum concentrations 2h after administration of insulin or LPS in both studies. This increase was greater after LPS treatment indicating that this may be a 'stronger' stressor than insulin. Interestingly, cortisol increased in an identical fashion between insulin-delayed and insulin-non-delayed animals in study 1. This unexpected finding provides direct evidence that cortisol is not a key disruptor of either the LH surge or sexual behaviour after insulin administration in intact ewes.

What neuronal pathways control sexual behaviour in the ewe?

ERα-cell activation in the VMN coincides with the exhibition of sexual behaviours supporting existing evidence that the VMN is involved in behavioural regulation (Chapter 4; Blache *et al.,* 1991). As 70% of ERα-containing cells in the VMN synthesise somatostatin (Herbison, 1995), it is plausible to speculate that this neuro-phenotype may be involved, but this requires further investigation. In Chapter 6, the percentage of activated dopamine neurones in the middle and caudal ARC was greater in the early follicular phase but decreased before and during sexual behaviour and the LH surge. It is, therefore, probable that progesterone, via dopamine, acts as a brake on the neuronal

circuits mediating the expression of sexual behaviour. Thereafter, increasing oestradiol concentrations overcome dopamine inhibition and sexual behaviour is initiated 12h later. Indeed, 20% of dopamine neurones provide input towards the VMN (Qi *et al.,* 2008) and could be a possible pathway for the control of female sexual behaviour. However, the cells that receive dopaminergic input within the VMN remain to be elucidated. It would be of great interest to determine whether somatostatin cells contain dopamine receptors and/or receive contact from dopaminergic fibres. Alternatively, as ERα-cells in the ARC become transcriptionally active before the activation of ERα-cells in the VMN and the concurrent exhibition of sexual behaviours, dopamine may act locally and transmit the disinhibiting signal to the VMN via unknown ARC inter-neurones.

What neuronal pathways control the GnRH surge in the ewe?

Activational patterns of neuro-phenotypes in the ARC at various stages during the follicular phase leading up to the GnRH/LH surge in intact ewes are shown in Fig. 1. In follicular phase ewes, ERα-cell activation was maximum during the LH surge in the ARC, VMN and mPOA indicating a role for these areas in oestradiol positive feedback and GnRH surge secretion. However, ERα-cells in the ARC are the first to become transcriptionally active (~6-7h before the expected surge onset), indicating that the surge induction process begins in this area, possibly when increasing oestradiol concentrations reach a specific 'threshold'. Signals may then be transmitted to the VMN and mPOA (Chapter 4). Chapter 5 provides evidence that this activation pattern in the ARC may reflect KNDy neurone activation and confirms that these cells play an important role in the generation of the GnRH surge mechanism. However, the balance of neuropeptide expression within this cell population appears to vary throughout the late follicular phase; initially (~6-7h before the expected surge onset), there is a shift of the net balance towards inhibitory dynorphin followed by a swing towards excitatory kisspeptin presumably facilitated by neurokinin B (during the surge). As KNDy cells send projections towards the mPOA (Lehman *et al.,* 2010) and ME (Amstalden *et al.,* 2005, Smith *et al.,* 2011), these signals may be directly or indirectly transmitted to GnRH cell bodies and/or terminals. Further studies, using *in situ* hybridisation at various times in the follicular phase to detect potential differences in the expression patterns of

dynorphin and kisspeptin, would be beneficial in furthering these findings. Other neuronal changes during the GnRH surge include a decrease in β-endorphin and an increase in NPY and somatostatin activation in the ARC as well as an increase in somatostatin in the VMN and kisspeptin in the mPOA. These results are, therefore, consistent with a role for kisspeptin during the surge but do not implicate this cell type in the early stages of surge activation. In contrast, dynorphin appears to have this role, as dynorphin containing cells are activated prior to the surge (but not during), and possibly constitutes a very early step in a chain of events leading to the GnRH surge in the ewe.

What neuronal pathways are activated for the disruption of sexual behaviour after the application of acute stressors in the late follicular phase?

Dopamine facilitates the expression of sexual behaviour while oestradiol concentrations are low but is inhibitory once oestradiol values increase above a threshold (Fabre-Nys *et al.,* 2004). This may constitute a potential pathway for the stress-induced suppression of sexual behaviour. However, LPS or insulin did not alter dopamine activation in the ARC and, therefore, it remains to be established whether other dopamine populations, such as those in the PVN, may account for the disruption. Cortisol increased to maximum concentrations immediately after the administration of LPS or insulin (i.e., 2h after treatment) and this steroid is, therefore, a potential candidate for the inhibition of sexual behaviours. However, cortisol is able to inhibit oestradiol-induced sexual receptivity (i.e., oestrus) but not other components of sexual behaviour that were also disturbed in the present studies (Pierce *et al.,* 2008, Papargiris *et al.,* 2011). Therefore, cortisol may contribute, but cannot be the sole mediator of stress-induced disruption of sexual behaviour. A small increase was observed in plasma progesterone concentrations after LPS (study 1 and 2) and insulin-delayed (study 1) animals. This subtle change may be the main mechanism by which all sexual behaviours were blocked (Scaramuzzi et al., 1971, Fabre-Nys 1998, Fabre-Nys and Gelez 2007). However, the neuroendocrine centres which are targets for progesterone, and are activated to inhibit sexual behaviour, remain to be elucidated. A potential role for dopamine is proposed. Performing microdialysis in the MBH during stress-induced disruption of sexual behaviour could shed some light on this aspect.

What neuronal pathways are activated for the disruption of the GnRH surge after the application of acute stressors in the late follicular phase?

The neuroendocrine alterations observed in the present studies after insulin or LPS are shown in Table 1. Insulin or LPS administration in the late follicular phase disturbed the majority of the neuronal activation patterns investigated. Our results suggest that these two stressors activate common as well as stress-specific inhibitory pathways. Interestingly, in study 2, some split responses were observed in certain parameters after insulin-treatment. As this division reduced the group size to n=2/group, statistical analysis was not undertaken, but the data are presented for information. Further studies with a larger number of animals should confirm these findings. The main split response observed involved two insulin-treated animals showing an intense c-Fos activation in the PVN (insulin-responders) whereas the other two were not different to controls (insulin-non-responders). A similar divergence was observed in study 1. Taking into account the fundamental importance of the PVN in response to stress (Antoni, 1986), it is speculated that the animals with no activation in the PVN would have gone on to have an LH surge at a similar time as the controls, whereas the others would have had a delayed surge. Even though the reasons for these split responses are not yet clear, this unexpected finding provides information about which alterations are involved in the stress-induced disruption of the LH surge. Interestingly, cortisol concentrations increased equally in both insulin sub-groups. The reason for this divergence is not known, and it is particularly interesting that even though PVN activation did not occur, plasma cortisol concentrations were elevated. In this aspect, Tilbrook and Clarke (2006) discuss the existence of individuals that are more sensitive to the negative feedback of glucocorticoids, resulting in stress hypo-responsiveness. Thus, insulin may be a less severe stressor than LPS, and certain individuals being more stress-resilient, are able to recover from the stress axis activation very quickly.

Our results indicate that KNDy cells are targets of immune/inflammatory and metabolic stressors. LPS treatment was accompanied by a failure of dynorphin neurone activation whereas, insulin prevented dynorphin activation in insulin-responders only. Dynorphin inhibition in the ARC is, therefore, a common parameter between LPS and insulin-responders and may be important in disrupting a necessary chain of events leading to

the GnRH surge. A split response was also observed in somatostatin activation in the VMN: these cells were activated in LPS and insulin-responders but not insulin-non-responders. In rats, somatostatin is one of the most potent inhibitors of electrical excitability of GnRH neurones identified thus far (Rotsztejn *et al.* 2002, Bhattarai *et al.,* 2010) and inhibits the LH surge when administered centrally (Van Vugt *et al.* 2003). It is, therefore, possible that somatostatin cells are activated via an unknown mechanism to mediate stress-induced disruption of the LH surge.

In the present study, we have shown, for the first time in the ewe, that there is an abundance of CRFR type 2 in the lower part of the ARC and the ME. In addition, 21% kisspeptin cells express this type of receptor and that doubled after LPS treatment. This indicates that CRFR type 2 may be involved in down-regulation of kisspeptin transcriptional activation. However, there was a large number of CRFR type 2 that co-localised with cells of unknown phenotype. There are, therefore, two possible pathways for CRF suppression of GnRH, one being the direct association of CRF and GnRH cell terminals in the external zone of the ME (Ghuman *et al.,* 2010) and the other being the regulation of kisspeptin and other cell types in the ARC and ME via CRFR type 2.

Interestingly, insulin increased kisspeptin, NPY, β-endorphin and somatostatin activation as well as overall c-Fos activation in the ARC in both sub-groups. This appears to be an insulin-specific response as the ARC plays a pivotal role in glucose-sensing and energy balance (Cone *et al.,* 2001; Routh, 2003). Reciprocal connections have been identified between kisspeptin-NPY-POMC cells (Backholer *et al.,* 2010). This network between the three cell types could co-ordinate reproduction and metabolic homeostatic systems. The present data suggest that somatostatin may also participate in this network. However, as these changes were observed in insulin-non-responders, they may not be disruptive to the LH surge.

Cortisol and progesterone suppress pulsatile GnRH/LH secretion (Karsch *et al.,* 1987, Debus *et al.,* 2002, Oakley *et al.,* 2009) and disrupt the positive feedback effect of oestradiol to trigger an LH surge (Kasa-Vubu *et al.,* 1992, Skinner *et al.,* 1998, Richter *et*

al., 2002, Smith *et al.,* 2003, Richter *et al.,* 2005, Pierce *et al.,* 2009, Wagenmaker *et al.,* 2009a). However, as mentioned above, cortisol responses were similar between insulin sub-groups in both studies, indicating that this steroid is not the key disruptor of the LH surge, at least after insulin treatment. The only observed hormonal difference between delayed and non-delayed groups was a subtle increase in plasma progesterone (Chapter 2), indicating that this may be an important inhibitory mechanism leading to the disruption of the LH surge.

Finally, it is clear that the approaches taken in this thesis have expanded understanding of events in both control and stressed ewes. Studying activation of several neuro-phenotypes at various times in the follicular phase with or without exposure to different stressors has made it possible to identify key steps in the control of stress-induced disruption of sexual behaviour and the GnRH/LH surge.

Fig. 1 Activational patterns of neuro-phenotypes in the ARC at various stages during the follicular phase relative to sexual behaviour and the GnRH/LH surge in intact ewes.

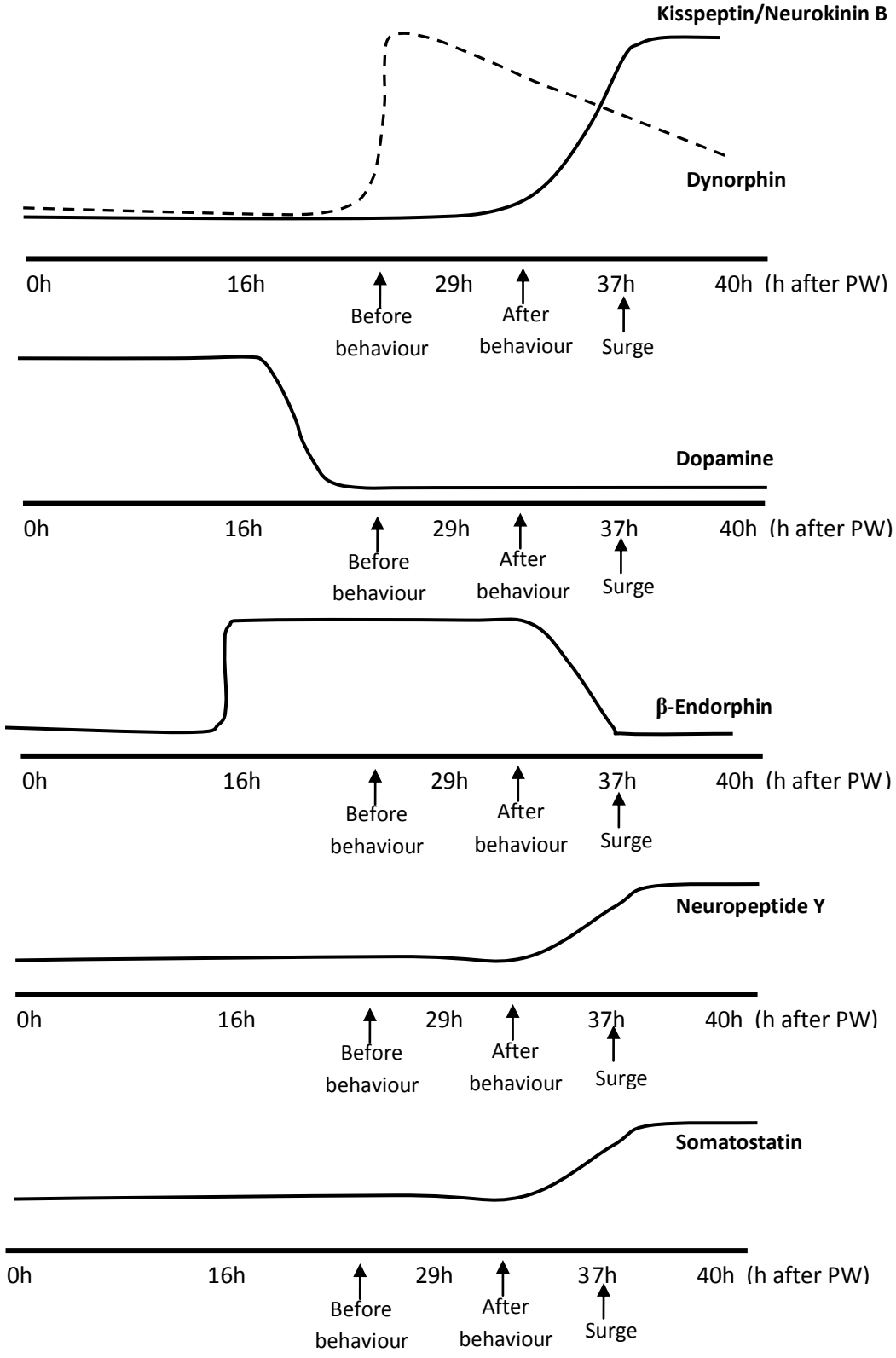

Table 1. Effect of insulin or LPS administration during the late follicular phase of intact ewes on various hormonal, behavioural and neuropeptide parameters.

Parameters	Insulin-non-responders	Insulin-responders	LPS
Sexual behaviour	=	delayed	delayed
LH surge	=	delayed	delayed
Oestradiol	↓ followed by a quick ↑	↓	↓
Progesterone	=	↑	↑↑
Cortisol	↑	↑	↑↑
Glucose	↓↓	↓↓	↓
c-Fos activation of the PVN	=	↑	↑↑
c-Fos activation of the ARC	↑	↑	↑
c-Fos activation of the VMN	=	↑	↑
c-Fos activation of the mPOA	=	=	↑
c-Fos activation of the ME	=	=	=
c-Fos activation of the BNST	=	=	=
c-Fos activation of the dBb	=	=	↑
% ERα-cell activation (ARC)	=	=	↓
% ERα-cell activation (VMN)	=	↑	=
% ERα-cell activation (mPOA)	=	↓	↓
% kisspeptin activation (ARC)*	↑	↑	↓
% kisspeptin activation (mPOA)*	=	=	↓
% dynorphin activation (ARC)	=	↓	↓
% neurokinin B activation (ARC)	=	=	=
% dopamine activation (ARC)	=	=	=
% β-endorphin activation (ARC)	↑	↑	=
% NPY activation (ARC)	↑	↑	=
% somatostatin activation (ARC)	↑	↑	=
% somatostatin activation (VMN)	=	↑↑	↑

Key: =no change compared to controls, ↓decrease compared to controls, ↑increase compared to controls. Two arrows indicate a greater response in the particular parameter compared to the other stressor. ** Decrease in the % kisspeptin activation was observed only 12h after LPS treatment, whereas there was no equivalent measurement for insulin-treated animals. Differences between insulin-responders or insulin-delayed and insulin-non-responders or insulin-non-delayed are highlighted for emphasis.

Appendix

Appendix

A) TISSUE PREPARATION-PERFUSION FIXATION

Perfusion-fixation allows tissue to be fixed via the vascular system. Tissue should be fixed within several minutes of anoxia to prevent antigen degradation and to provide optimal staining. The procedure commonly washes blood from the vasculature before introducing the fixative.

Method of perfusion fixation.

1. **Euthanasia:** 20ml pentobarbital plus 25,000iu heparin (1 vial).
2. **Head retrieval:** Each head was retrieved immediately, jugular veins occluded and perfused bilaterally via the carotid arteries using a peristaltic pump.
3. **Flush/blood vessel dilation:** A total of 2 litres comprising:
    - 2 litres phosphate buffer (pH 7.4; see later)
    - + 25,000iu heparin/ml (2 vials)
    - + 1% sodium nitrite.
4. **Zamboni's fixative:** Total 2 litres comprising:
    - 200ml paraformaldehyde (4%; powder form)
    - + 150 ml picric acid (7.5%)
    - + 1650 phosphate buffer
5. **Zamboni's + sucrose flush:** Total 0.5 litres comprising:
    - 0.5 litres Zamboni's fixative
    - + 150 g sucrose (30%)
6. The brain was left within the skull for a further 4 h
7. **PB sucrose wash-out:** Total 0.5 litres comprising:
    - 0.5 litres phosphate buffer
    - + 200g sucrose (40%)
    - + 0.5g sodium azide (0.1 %)
8. **Dissect** out the brain, cutting the cranial nerves

9. **Retrieve a 17mm hypothalamic block** (extending from the optic chiasma to the mammilary bodies).
10. **Immerse tissue in wash-out solution:** at 4^0C for a week.
11. **Freeze hypothalamic blocks** using isopentane and liquid nitrogen as described by Rosene et al. (1986)
12. **Store** at -80^0C.

Method of tissue sectioning

- Thaw hypothalamic blocks in wash-out solution.
- Place the tissue on the stage of the microtome and freeze with dry ice. Section at a thickness of 40μm and collect the sections from the knife blade with a buffer-moistened brush.
- Store sections in 96-well microtest plates with each section placed in an individual well filled with cryoprotectant solution (see later for recipe).
- Store at -80^0C until processed for immunohistochemistry.

B) <u>IMMUNOSTAINING TECHNIQUES</u>

Immunohistochemistry, enzyme method for free-floating sections.

1. **Wash in PBS:** for 2h and 4 changes. This wash is important because it removes cryoprotectant solution.
2. **Peroxidase Blocking:** 1% H2O2, 40% methanol, in PBS.
   For 15 min, in room temperature.
   <u>For example: to make up 250 ml</u>
   100 ml methanol
   8.35ml of H2O2 30% solution
   141.65 ml of PBS.
3. **Wash in PBS:** 3 changes 5 minutes each
4. **Block with donkey serum:** 10% in PBS.
   For 1h at room temperature. During this step primary antibody dilution can be done, ready to apply when this incubation is finished.

For example: to make up 50 ml

5ml donkey serum

in 45 ml PBS**.**

5. **Incubation in primary.**

   A list of primary antibodies and dilutions are shown in Table 1.

   For 3 days in 4^0C.

   Dilute in antibody diluting solution (recipe below). If double-staining then always apply the nuclear stain first (i.e. c-Fos or ERα).

6. **Wash in PBS:** for 2h and 4 changes. This wash is important because it removes unbound antibody.

7. **Incubate in biotinylated donkey anti-rabbit IgG:**

   (Anti-Rb) if primary is raised in rabbit or (Anti-Mo) if primary is raised in mouse.

   Dilution 1:500. Adjust accordingly if background is too high or immunostaining too weak. Dilute in antibody dilution solution (recipe below).

   For 2h at room temperature.

   Make up your ABC solution for next step.

8. **Wash in PBS:** 3 changes 5 minutes each

9. **Incubate in Vectastain Elite ABC kit**.

   Needs to be made up at least half an hour before applied.

   Usual dilution 1:250. Adjust accordingly if background is too high or immunostaining too weak. Dilute in PBS.

   For 1.5h in room temperature. There are 2 solutions A and B put in equal amounts and dilute in PBS.

10. **Wash in PBS:** 3 changes 5 minutes each.

11. **Apply ni-DAB (black reaction product) or DAB alone (brown reaction product).**

    Make up according to instructions.

    Incubate for 5min at room temperature.

12. **Wash in PBS:** 3 changes 5 minutes each.

    Last wash in distilled water.

13. **Repeat from peroxidase blocking step, to stain for the second antibody or continue to next step if staining is completed.**

14. **Mount sections:** on chrome alum gelatine coated slides (recipe below).

15. **Dry sections:** for 3 days, covered to avoid dusting.
16. **Dehydrate in a series of solutions. Ethanol diluted in distilled water: 70%, 85%, 96%, 100%, 100%, 100%, then Xylene.**
    For 5 min in each solution. Carried out under a fume cupboard.
17. **Cover slip in DPX and** store in room temperature.

Immunofluorescence for free-floating sections.

1. **Wash in PBS:** for 2h and 4 changes. This wash is important because it removes cryoprotectant solution.
2. **Block with donkey serum:** 10% in PBS.
   For 1h at room temperature. During this step primary antibody dilution can be done, ready to apply when this incubation is finished.
   For example: to make up 50 ml
   5ml donkey serum
   in 45 ml PBS**.**
3. **Incubation in primary.**
   A list of primary antibodies and dilutions are shown in Table 1.
   For 3 days in 4^0C.
   Dilute in antibody diluting solution (recipe below). If double-staining then always apply the nuclear stain first (i.e. c-Fos or ERα).
4. **Wash in PBS:** for 2h and 4 changes. This wash is important because it removes unbound antibody.
5. **Incubate in secondary antibody: From here onwards cover to protect from light.**
   (Anti-Rb) if primary is raised in rabbit or (Anti-Mo) if primary is raised in mouse. Dilution 1:500. Adjust accordingly if background is too high or immunostaining too weak. Dilute in antibody dilution solution (recipe above).
   For 2h at room temperature.
6. **Repeat from first washing step, to stain for the second antibody or continue to next step if staining is completed.**

7. **Wash in PBS:** 3 changes 5 minutes each.
   Last wash in distilled water.
8. **Mount sections:** on chrome alum gelatine coated slides (recipe below).
9. **Dry sections:** for 10-20 min
10. **Cover slip with Anti-fade mounting medium** and store in 4^0C.

Triple Staining Method; combination of DAB and fluorescence staining for free-floating sections.

This method consisted of an immunoperoxidase protocol in which nuclear c-*F*os was detected first with diaminobenzidine as chromogen (DAB; brown reaction product) followed by visualisation of dynorphin and kisspeptin with immunofluorescence. As dynorphin and kisspeptin antibodies were of rabbit origin, we used a previously described modified protocol (Hunyady *et al.,* 1996, Goodman *et al.,* 2007, Cheng *et al.,* 2010). In brief, the first antigen was visualised using a very low concentration of primary antibody with tyramide amplification solution (TSA). Next, the second antigen was visualised using normal concentrations of primary antibody and detection with fluorophore-conjugated secondary antibody.

1. **Wash in PBS:** for 2h and 4 changes. This wash is important because it removes cryoprotectant solution.
2. **Peroxidase Blocking:** 1% H2O2, 40% methanol, in PBS.
   For 15 min, in room temperature.
   <u>For example: to make up 250 ml</u>
   100 ml methanol
   8.35ml of H2O2 30% solution
   141.65 ml of PBS.
3. **Wash in PBS:** 3 changes 5 minutes each
4. **Block with donkey serum:** 10% in PBS.
   For 1h at room temperature. During this step primary antibody dilution can be done, ready to apply when this incubation is finished.

For example: to make up 50 ml

5ml donkey serum

in 45 ml PBS**.**

5. **Incubation in Anti-c-*Fos*.**

   Recommended dilution is 1:5000. For 3 days in 4^0C.

   Dilute in antibody diluting solution (recipe below).

6. **Wash in PBS:** for 2h and 4 changes. This wash is important because it removes unbound antibody.

7. **Incubate in biotinylated donkey anti-rabbit IgG:**

   Dilution 1:500. Adjust accordingly if background is too high or immunostaining too weak. Dilute in antibody dilution solution (recipe below).

   For 2h at room temperature.

   Make up your ABC solution for next step.

8. **Wash in PBS:** 3 changes 5 minutes each

9. **Incubate in Vectastain Elite ABC kit**.

   Needs to be made up at least half an hour before applied.

   Usual dilution 1:250. Adjust accordingly if background is too high or immunostaining too weak. Dilute in PBS.

   For 1.5h in room temperature. There are 2 solutions A and B put in equal amounts and dilute in PBS.

10. **Wash in PBS:** 3 changes 5 minutes each.

11. **Apply ni-DAB.**

    Make up according to instructions.

    Incubate for 5min in room temperature.

12. **Wash in PBS:** 3 changes 5 minutes each.

13. **Repeat steps 2-4.**

14. **Incubate in Anti-kisspeptin.**

    Recommended dilution is 1:150000. For 3 days in 4^0C.

    Dilute in antibody diluting solution (recipe below).

15. **Repeat steps 7-10.**

16. **Incubate in TSA.**

    This step amplifies the kisspeptin protein signal.

Recommended dilution 1:200. For 10min in room temperature.

Dilute in PBS with 0.003% H_2O_2 as substrate.

17. **Incubate in Streptavidin conjugated fluorophore. From here onwards cover to protect from light.**

    Recommended: Streptavidin Conjugated AlexaFluor 488 at a dilution of 1:100 for 2h.

18. **Repeat** steps 3-4.
19. **Incubate in Anti-dynorphin.**

    Recommended dilution is 1:10000. For 3 days in 4^0C.

    Dilute in antibody diluting solution (recipe below).

20. **Wash in PBS:** 3 changes 20 minutes each.
21. **Incubate in secondary fluorophore.**

    Recommended: Cy3 at a dilution of 1:500 for 2h.

22. **Wash in PBS:** 3 changes 5 minutes each.

    Last wash in distilled water.

23. **Mount sections:** on chrome alum gelatine coated slides (recipe below).
24. **Dry sections:** for 10-20 min
25. **Cover slip with Anti-fade mounting medium** and store in 4^0C.

Table 1. Dilution, source of purchase and references, that verify use in ovine neuronal tissue, of primary antibodies used in the present thesis.

Antibody	Host	Dilution	Source	Reference
Anti-c-Fos	Rabbit	1:5000	Calbiochem	Ghuman *et al.,* 2011
Anti-ERα	Mouse	1:50	DAKO	Franceschini *et al.,* 2006
Anti-TH	Mouse	1:20000	Millipore	Robinson *et al.,* 2010
Anti-NPY	Rabbit	1:5000	Sigma	Skinner and Herbison, 1997
Anti-Kisspeptin	Rabbit	1:25000	Prof. Alain Caraty	Franceschini *et al.,* 2006
Anti-Dynorphin A	Rabbit	1:10000	Peninsula Laboratories	Goodman *et al.,* 2007
Anti-Neurokinin B	Rabbit	1:1000	Peninsula Laboratories	Goodman *et al.,* 2007
Anti-Somatostatin	Rabbit	1:500	Peninsula Laboratories	Robinson *et al.,* 2010
Anti-β-Endorphin	Rabbit	1:500	Peninsula Laboratories	Ghuman *et al.,* 2011
Anti-CRFR type 2	Rabbit	1:4000	Abcam	Lakshmanan *et al.* 2007

C) BUFFERS AND SOLUTIONS

Sodium Phosphate Buffer (PB 0.1M)

This buffer was made by mixing the two following stock solutions:

Stock A

0.2M Sodium di-hydrogen orthophosphate 1-hydrate

[Dissolve 27.6 gr NaH_2PO_4 $1H_2O$ (Mol. Wt. = 137.99)/litre of distilled water]

Stock B

0.2M Di-sodium hydrogen phosphate (anhydrous)

[Dissolve 28.4 gr Na_2HPO_4 (Mol. Wt. = 142)/litre of distilled water]

Mixing System

For pH 7.2 sodium phosphate buffer, mix:

Volume required (ml)	Stock A (ml)	Stock B (ml)	Distilled water (ml)
100	14	36	50
1000	140	360	500
3000	420	1080	1500
5000	700	1800	2500

Phosphate Buffered Saline (PBS 0.1M)

This buffer solution was made up by adding 0.9% Sodium Chloride (NaCl) to the PB solution as prepared above.

Cryoprotectant solution

To make 1l stir and dissolve the following:

- 500 ml PB (0.1M)

- 300gr Sucrose. Stir to dissolve.
- 10gr Polyvinylpyrrolidone (PVP-40). Stir to dissolve.
- 300ml ethylene glycol. Stir to dissolve.

Adjust final volume to 1000ml with distilled water.

Antibody Diluting Solution

All antibodies were diluted with the following solution:

- 2.5% Normal Donkey Serum
- 1% Triton
- 0.25% Sodium Azide

} Diluted in PBS (0.1M)

A large volume of this solution can be made and stored at 4^{o}C.

Chrome Alum Gelatine Coating for slides

- Add 1gr Gelatine in 200 ml distilled water. Warm and stir to dissolve
- Add 0.19gr Chrome Alum. Stir to dissolve.
- Add 0.5gr Sodium Azide. Stir to dissolve.
- Filter and store at room temperature.

Dip clean (grease free) slides in the solution and leave to dry at room temperature for about 6 h, covering to keep dust-free. Once dried, slides can be packed together and this will not damage the coating.

D) SHEEP BRAIN ATLAS

Cressyl Fast Violet

Preparation of stain

Add 0.25gr of Cressyl fast violet in 100 ml of distilled water. The solution was dissolved by stirring and heating and then filtered. 5 drops of acetic acid was added to 100 ml of solution.

Method

Slides were left in the above solution for 10 minutes in an oven at 60^0C. They were then rinsed in distilled water, differentiated in 96%, 100% alcohol and cleared in xylene. Finally slides were cover slipped with DPX.

Sample sections from the sheep brain atlas are shown in Fig. 1. For the whole atlas please refer to the C.D. accompanied with the thesis.

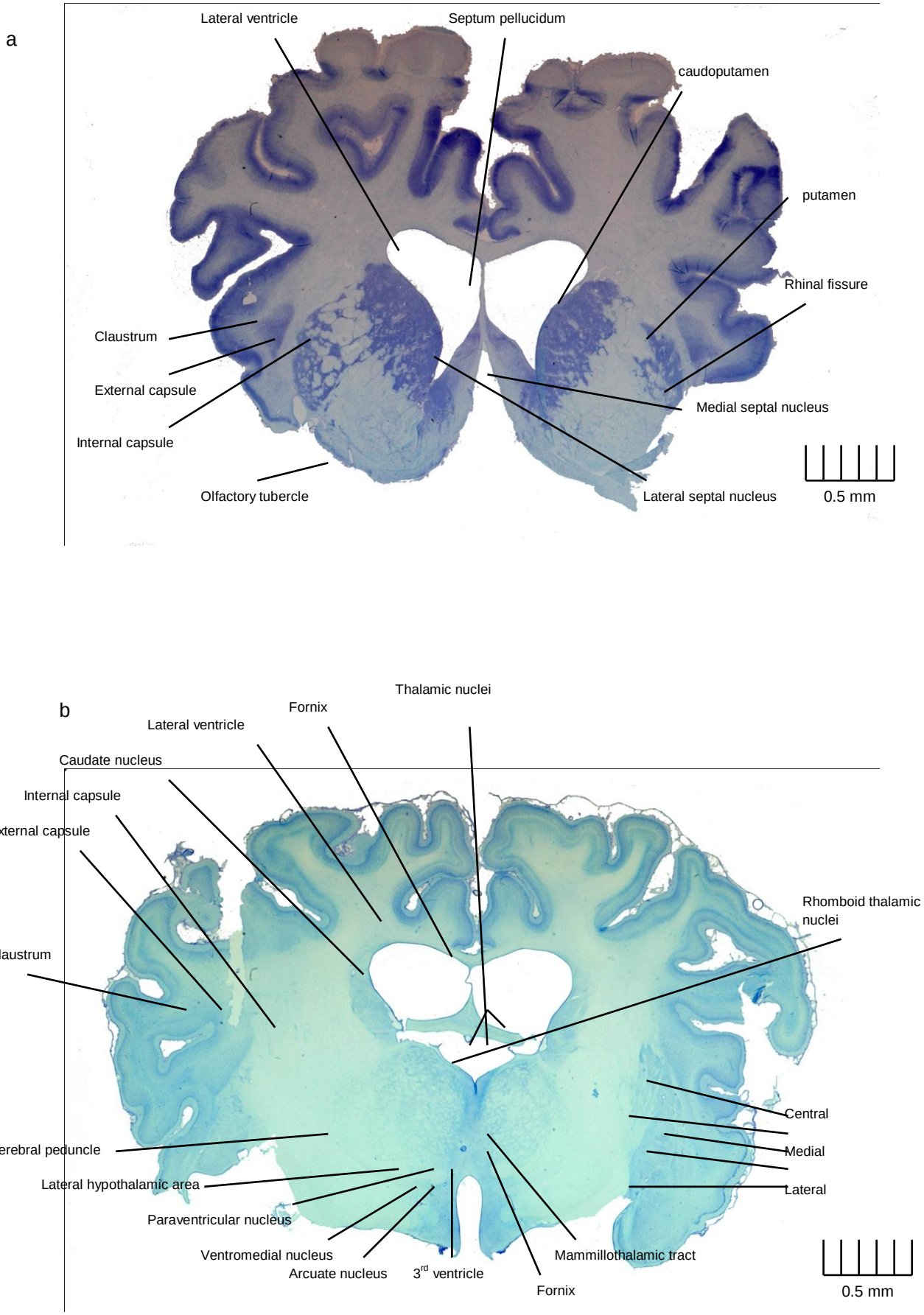

Fig. 1 Sections 05 (a) and 28 (b) of the sheep brain atlas. The atlas consists of 34 consecutive sections, 50 µm thick and 0.5 mm apart, extending from the optic chiasma to the mammilary bodies.

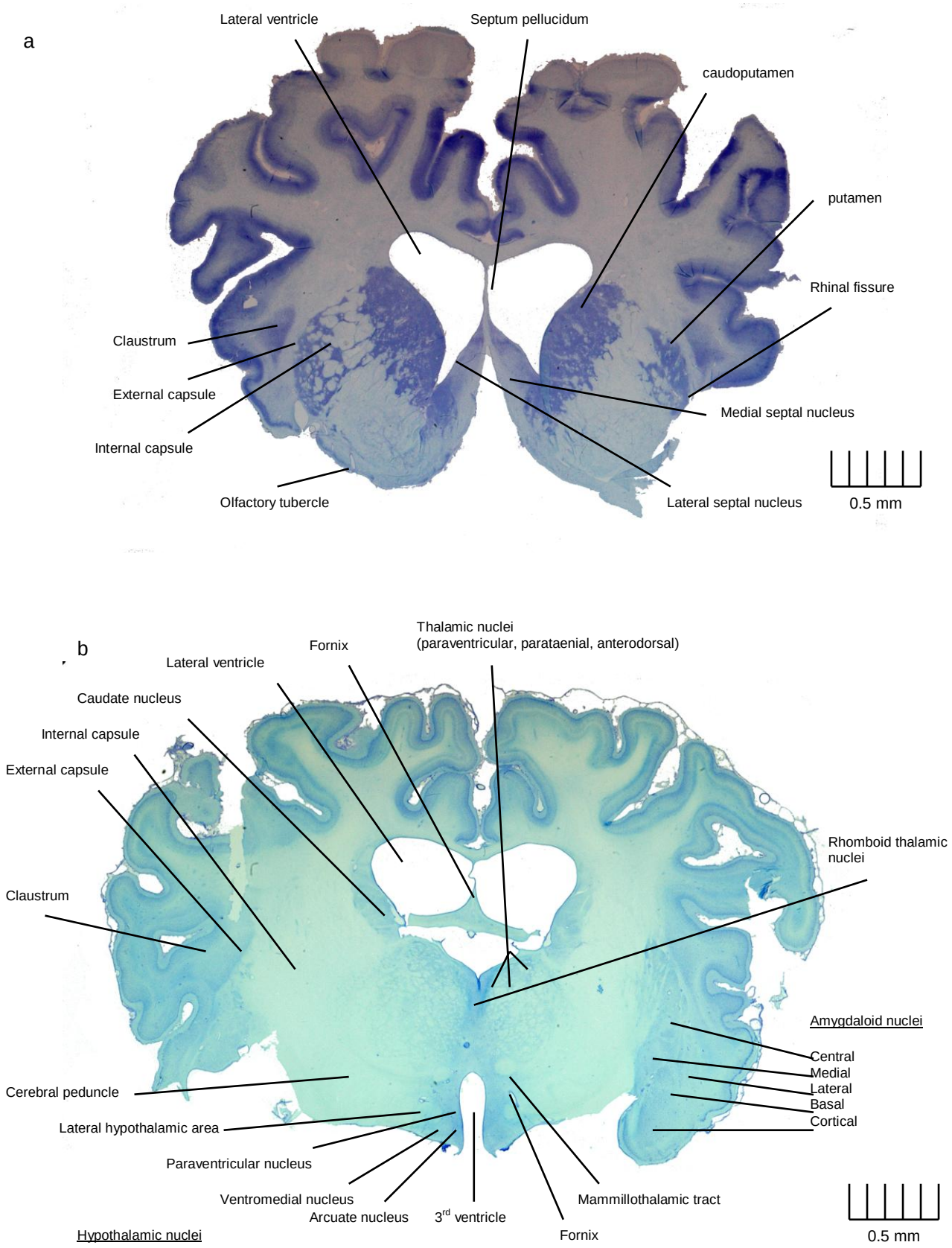

Fig. 1 Sections 05 (a) and 28 (b) of the sheep brain atlas. The atlas consists of 34 consecutive sections, 50 µm thick and 0.5 mm apart, extending from the optic chiasma to the mammilary bodies.

Bibliography

Bibliography

Adachi, S., Yamada, S., Takatsu, Y., Matsui. H., Kinoshita. M., Takase, K., Sugiura, H., Ohtaki. T., Matsumoto, H., Uenoyama, Y., Tsukamura, H., Inoue, K, Maeda, K. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev. 2007, 53:367-78.

Adam C. L., Findlay, P. A., Kyle, C. E., Young, P., Mercer, J. G. Effect on chronic food restriction on pulsatile luteinizing hormone secretion and hypothalamic neuropeptide Y gene expression in castrate male sheep. J of Endocrinol. 1997, **152:**329-37.

Advis, J. P., Klein, J., Kuljis, R. O., Sarkar, D. K., McDonald, J. M., Conover, C. A. Regulation of gonadotropin releasing hormone release by neuropeptide Y at the median eminence during the preovulatory period in ewes. Neuroendocrinology. 2003,77:246-57.

Alexander, M. J. Estrogen-regulated synthesis of neurotensin in neurosecretory cells of the hypothalamic arcuate nucleus in the female rat. Endocrinology. 1993,133:1809-16.

Alexander, M. J., Leeman, S. E. Neurotensin gene expression in the rat preoptic area. Implications for the regulation of reproduction. Ann N Y Acad Sci. 1992,668:70-89.

Amstalden, M., Coolen, L. M., Hemmerle, A. M., Billings, H. J., Connors, J. M., Goodman, R. L., Lehman, M. N. Neurokinin 3 receptor immunoreactivity in the septal region, preoptic area and hypothalamus of the female sheep: colocalisation in neurokinin B cells of the arcuate nucleus but not in gonadotrophin-releasing hormone neurones. J Neuroendocrinology. 2010,22:1-12.

Anderson, G. M., Connors, J. M., Hardy, S. L., Valent, M., Goodman, R. L. Oestradiol microimplants in the ventromedial preoptic area inhibit secretion of luteinizing hormone via dopamine neurones in anoestrous ewes. J Neuroendocrinology.

2001,13:1051-8.

Antoni, F. A. Hypothalamic control of adrenocorticotrophin secretion: advances since the discovery of 41 residue CRF. Endocrine Reviews. 1986, 7:351-78.

Antonopoulos, J., Papadopoulos, G. C., Karamanlidis, A. N, Michaloudi, H. Distribution of neuropeptides in the infundibular nucleus of the sheep. Neuropeptides. 1989, 14:121-28.

Baldino, F., Jr., Fitzpatrick-McElligott, S., O'Kane, T. M., Gozes, I. Hormonal regulation of somatostatin messenger RNA. Synapse. 1988,2:317-25.

Barraclough, C. A., Wise, P. M. The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. Endocrine reviews. 1982,3:91-119.

Barrell, G. K., Moenter, S. M., Caraty, A., Karsch, F. J. Seasonal changes of gonadotropin-releasing hormone secretion in the ewe. Biology of Reproduction. 1992,46:1130-5.

Batailler, M., Blache, D., Thibault, J., Tillet, Y. Immunohistochemical colocalization of tyrosine hydroxylase and estradiol receptors in the sheep arcuate nucleus. Neuroscience letters. 1992,146:125-30.

Battaglia, D. F., Beaver, A. B., Harris, T. G., Tanhehco, E., Viguie, C., Karsch, F. J. Endotoxin disrupts the estradiol-induced luteinizing hormone surge: interference with estradiol signal reading, not surge release. Endocrinology. 1999,140:2471–79

Battaglia, D. F., Brown, M. E., Krasa, H. B., Thrun, L. A., Viguie, C., Karsch, F. J. Systemic challenge with endotoxin stimulates corticotropin-releasing hormone and arginine vasopressin secretion into hypophyseal portal blood: coincidence with gonadotropin-releasing hormone suppression. Endocrinology. 1998, 139:4175-81.

Battaglia, D. F., Krasa, H. B., Padmanabhan, V., Viguie, C., Karsch, F. J. Endocrine alterations that underlie endotoxin-induced disruption of the follicular phase in ewes. Biology of Reproduction. 2000, 62:45-53.

Battaglia, D. F., Bowen, J. M., Krasa, H. B., Thrun, L. A., Viguie, C., Karsch, F. J. Endotoxin inhibits the reproductive neuroendocrine axis while stimulating adrenal steroids: a simultaneous view from hypophyseal portal and peripheral blood. Endocrinology. 1997,138:4273-81.

Beach, F. A. Sexual attractivity, proceptivity, and receptivity in female mammals. Horm Behav. 1976, 7**:**105-38.

Ben Said, S., Lomet, D., Chesneau, D., Lardic, L., Canepa, S., Guillaume, D., Briant, C., Fabre-Nys, C., Caraty, A. Differential estradiol requirement for the induction of estrus behavior and the luteinizing hormone surge in two breeds of sheep. 2007, Biology of Reproduction. 76**:**673-80.

Bethea, C. L., Centeno, M. L., Cameron, J. L. Neurobiology of stress-induced reproductive dysfunction in female macaques. Mol Neurobiol. 2008, 38**:**199-230.

Bertrand, F., Viguie, C., Picard, S., Malpaux, B. Median eminence dopaminergic activation is critical for the early long-day inhibition of luteinizing hormone secretion in the ewe. Endocrinology. 1998,139:5094-102.

Bhattarai, J. P., Kaszas, A., Park, S. A., Yin, H., Park, S. J., Herbison, A. E., Han, S. K., Abraham, I. M. Somatostatin inhibition of gonadotropin-releasing hormone neurons in female and male mice. Endocrinology. 2010,151:3258-66.

Billings, H. J., Connors, J. M., Altman, S. N., Hileman, S. M., Holaskova, I., Lehman, M. N., McManus, C. J., Nestor, C. C., Jacobs, B. H., Goodman, R. L. Neurokinin B acts via the neurokinin-3 receptor in the retrochiasmatic area to stimulate luteinizing hormone secretion in sheep. Endocrinology. 2010,151:3836-46.

Blache, D., Batailler, M., Fabre-Nys, C. Oestrogen receptors in the preoptico-hypothalamic continuum: immunohistochemical study of the distribution and cell density during induced oestrous cycle in ovariectomized ewe. J Neuroendocrinology. 1994,6:329-39.

Blache, D., Fabre-Nys, C. J., Venier, G. Ventromedial hypothalamus as a target for

oestradiol action on proceptivity, receptivity and luteinizing hormone surge of the ewe. Brain Res. 1991, 546:241-9.

Bormann, J. The 'ABC' of GABA receptors. Trends Pharmacol Sci. 2000,21:16-9.

Bowen, J. M., Dahl, G. E., Evans, N. P., Thrun, L. A., Wang, Y., Brown, M. B., Karsch, F. J. Importance of the gonadotropin-releasing hormone (GnRH) surge for induction of the preovulatory luteinizing hormone surge of the ewe: dose-response relationship and excess of GnRH. Endocrinology. 1998,139:588-95.

Brailoiu, G. C., Dun, S. L., Ohsawa, M., Yin, D., Yang, J., Chang, J. K., Brailoiu, E., Dun, N. J. KiSS-1 expression and metastin-like immunoreactivity in the rat brain. The Journal of comparative neurology. 2005,481:314-29.

Brann, D. W., Mahesh, V. B. Excitatory amino acids: evidence for a role in the control of reproduction and anterior pituitary hormone secretion. Endocrine Reviews. 1997, 18:678–700.

Breen, K. M., Billings, H. J., Debus, N., Karsch, F. J. Endotoxin inhibits the surge secretion of gonadotropin-releasing hormone via a prostaglandin-independent pathway. Endocrinology. 2004,145:221-7.

Briski, K. P., Nedungadi, T. P., Koshy Cherian, A. Effects of hypoglycaemia on neurotransmitter and hormone receptor gene expression in laser-dissected arcuate neuropeptide Y/agouti-related peptide neurones. J Neuroendocrinol. 2010,22:599-607.

Brothers, K. J., Wu, S., DiVall, S. A., Messmer, M. R., Kahn, C. R., Miller, R. S., Radovick, S., Wondisford, F. E., Wolfe, A. Rescue of obesity-induced infertility in female mice due to a pituitary specific knockout of the insulin receptor. Cell Metabolism. 2010, 12:295–305.

Bruneau G, Tillet Y. Localization of the preprosomatostatin-mRNA by in situ hybridization in the ewe hypothalamus. Peptides 1998; 19: 1749–1758.

Caraty, A., Delaleu, B., Chesneau, D., Fabre-Nys, C. Sequential role of E2 and GnRH for

the expression of estrous behavior in ewes. Endocrinology. 2002, 143:139-45.

Caraty, A., Fabre-Nys, C., Delaleu, B., Locatelli, A., Bruneau, G., Karsch, F. J., Herbison, A. 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. 1998, 139**:**1752-60.

Caraty, A., Franceschini, I., Hoffman, G. E. Kisspeptin and the preovulatory gonadotrophin-releasing hormone/luteinising hormone surge in the ewe: basic aspects and potential applications in the control of ovulation. J Neuroendocrinology. 2010, 22:710-5.

Caraty, A., Grino, M., Locatelli, A., Guillaume, V., Boudouresque, F., Conte-Devolx, B., Oliver, C. Insulin-induced hypoglycemia stimulates corticotropin-releasing factor and arginine vasopressin secretion into hypophysial portal blood of conscious, unrestrained rams. J Clin Invest. 1990, 85:1716-21.

Caraty, A., Locatelli, A., Martin, G. B. Biphasic response in the secretion of gonadotrophin-releasing hormone in ovariectomized ewes injected with oestradiol. Journal of Endocrinology. 1989, 123:375-82.

Caraty, A., Miller, D. W., Delaleu, B., Martin, G. B. Stimulation of LH secretion in sheep by central administration of corticotrophin-releasing hormone. J Reprod Fertil. 1997, 111:249-57.

Cates, P. S., Li, X. F., O'Byrne, K. T. The influence of 17beta-oestradiol on corticotrophin-releasing hormone induced suppression of luteinising hormone pulses and the role of CRH in hypoglycaemic stress-induced suppression of pulsatile LH secretion in the female rat. Stress. 2004, 7:113-8.

Castellano, J. M., Navarro, V. M., Fernandez-Fernandez, R., Nogueiras, R., Tovar, S., Roa, J., Vazquez, M. J., Vigo, E., Casanueva, F. F., Aguilar, E., Pinilla, L., Dieguez, C., Tena-Sempere, M. Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology. 2005,146:3917-25.

Castellano, J. M., Roa, J., Luque, R. M., Dieguez, C., Aguilar, E., Pinilla, L., Tena-Sempere, M. KiSS-1/kisspeptins and the metabolic control of reproduction: physiologic roles and putative physiopathological implications. Peptides. 2009, 30: 139-45.

Chaillou, E., Baumont, R., Tramu, G., Tillet, Y. Long-term undernutrition followed by short-term refeeding effects on the corticotropin-releasing hormone containing neurones in the paraventricular nucleus: an immunohistochemical study in sheep. J Neuroendocrinology. 2002,14:269-75.

Chaillou, E., Tramu, G., Tillet, Y. Distribution of galanin immunoreactivity in the sheep diencephalon. J Chem Neuroanat. 1999,17:129-46.

Chalmers, D. T., Lovenberg, T. W., De Souza, E. B. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci. 1995, 15:6340–50.

Cheng, G., Coolen, L. M., Padmanabhan, V., Goodman, R. L., Lehman, M. N. The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. Endocrinology. 2010, 151:301-11.

Clarke, I. J. GnRH and ovarian hormone feedback. Oxf Rev Reprod Biol. 1987,9:54-95.

Clarke, I. J., Cummins, J. T., Crowder, M. E., Nett, T. M. Pituitary receptors for gonadotropin-releasing hormone in relation to changes in pituitary and plasma luteinizing hormone in ovariectomized-hypothalamo pituitary disconnected ewes. I. Effect of changing frequency of gonadotropin-releasing hormone pulses. Biology of Reproduction 1987,37:749-54.

Clarke, I. J. Control of GnRH secretion. Journal of reproduction and fertility. Supplement. 1987,34:1-8.

Clarke, I. J., Cummins, J. T. Direct pituitary effects of estrogen and progesterone on

gonadotropin secretion in the ovariectomized ewe. Neuroendocrinology. 1984,39:267-74.

Clarke, I.J., Horton, R.J., Doughton, B.W. Investigation of the mechanism by which insulin-induced hypoglycemia decreases luteinizing hormone secretion in ovariectomized ewes. Endocrinology. 1990, 127:1470–76.

Clarke, I. J., Pompolo. S., Scott, C. J., Rawson, J. A., Caddy, D., Jakubowska, A. E., Pereira, A. M. Cells of the arcuate nucleus and ventromedial nucleus of the ovariectomized ewe that respond to oestrogen: a study using Fos immunohistochemistry. J Neuroendocrinology. 2001, **13**:934–41

Clarkson, J., d'Anglemont de Tassigny, X., Moreno, A. S., Colledge, W. H., Herbison, A. E. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. J Neurosci. 2008, 28, 8691-7.

Clarkson, J., Herbison, A. E. Oestrogen, kisspeptin, GPR54 and the pre-ovulatory luteinising hormone surge. J Neuroendocrinology. 2009, 21:305-11.

Clarkson, J., Herbison, A. E. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology. 2006,147:5817-25.

Colthorpe, K. L., Curlewis, J. D. Localization and characterization of dopamine D1 receptors in sheep hypothalamus and striatum. J Neuroendocrinology. 1996,8:561-8.

Cone, R.D., Cowley, M.A., Butler, A.A., Fan, W., Marks, D.L., Low, M.J. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int J Obes Relat Metab Disord. 2001, 25:563–67.

Cordoba-Chacon, J., Gahete, M. D., Castano, J. P., Kineman, R. D., Luque, R. M. Somatostatin and its receptors contribute in a tissue-specific manner to the sex-dependent metabolic (fed/fasting) control of growth hormone axis in mice. Am J

Physiol Endocrinol Metab. 2011,300:E46-54.

Cunningham. M. J., Clifton, D. K., Steiner, R. A. Leptin's actions on the reproductive axis: perspectives and mechanisms. Biology of Reproduction 1999, 60:216–22.

d'Anglemont de Tassigny, X., Fagg,L.A., Carlton, M. B. L., Colledge, W.H. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology. 2008, 149:3926–32.

Debus, N., Breen, K. M., Barrell, G. K., Billings, H. J., Brown, M., Young, E. A., Karsch, F. J. Does cortisol mediate endotoxin-induced inhibition of pulsatile luteinizing hormone and gonadotropin-releasing hormone secretion? Endocrinology. 2002,143:3748-58.

DeFazio, R. A., Heger, S., Ojeda, S. R., Moenter, S. M. Activation of A-type gamma-aminobutyric acid receptors excites gonadotropin-releasing hormone neurons. Mol Endocrinol. 2002,16:2872-91.

de Greef, W. J., Eilers, G. A., de Koning, J., Karels, B., de Jong, F. H. Effects of ovarian inhibin on pulsatile release of gonadotrophins and secretion of LHRH in ovariectomized rats: evidence against a central action of inhibin. J Endocrinol. 1987,113:449-55.

Dhillo, W. S., Murphy, K. G., Bloom, S. R. The neuroendocrine physiology of kisspeptin in the human. Rev Endocr Metab Disord. 2007, 8:41-6.

Dhillo, W. S., Chaudhri, O. B., Patterson, M., Thompson, E. L., Murphy, K. G., Badman, M. K., McGowan, B. M., Amber, V., Patel, S., Ghatei, M. A., Bloom, S. R. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. The Journal of clinical endocrinology and metabolism. 2005,90:6609-15.

Dobson, H., Ghuman, S., Prabhakar, S., Smith, R. A conceptual model of the influence of stress on female reproduction. Reproduction. 2003, 125**:**151-63.

Dobson, H., Smith, R. F. What is stress, and how does it affect reproduction? Anim Reprod Sci. 2000, 61**:**743-52.

Dobson, H., Tebble, J. E., Ozturk, M., Smith, R. F. Effect of transport on pulsatile LH release in ovariectomized ewes with or without prior steroid exposure at different times of year. J Reprod Fertil. 1999, 117**:**213-22.

Domanski, E., Chomicka, L. K., Ostrowska, A., Gajewska, A., Mateusiak, K. Release of luteinizing hormone-releasing hormone, beta-endorphin and noradrenaline by the nucleus infundibularis/median eminence during periovulatory period in the sheep. Neuroendocrinology. 1991,54:151-8.

Doney, J. M., Gunn, R. G., Griffiths, J. G. The effect of premating stress on the onset of oestrus and on ovulation rate in Scottish Blackface ewes. J Reprod Fertil. 1973, 35**:**381-4.

Downing, J. A., Joss, J., Scaramuzzi, R. J. The effect of a direct arterial infusion of insulin and glucose on the ovarian secretion rates of androstenedione and oestradiol in ewes with an autotransplanted ovary. J Endocrinol. 1999, 163**:**531-41.

Dufourny, L., Schofield, N., Skinner, D. C. Immunoreactive galanin expression in ovine gonadotropin-releasing hormone neurones: no effects of gender or reproductive status. J Neuroendocrinology. 2003,15:1062-9.

Dufourny, L., Caraty, A., Clarke, I. J., Robinson, J. E., Skinner, D. C. 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 – A retrograde labeling study. Neuroendocrinology. 2005, 81:139-149.

Dufourny, L., Skinner, D. C. Progesterone receptor, estrogen receptor alpha, and the type II glucocorticoid receptor are coexpressed in the same neurons of the ovine preoptic area and arcuate nucleus: a triple immunolabeling study. Biology of Reproduction. 2002,67:1605-12.

Dufourny, L., Warembourg, M. Estrogen modulation of neuropeptides: somatostatin, neurotensin and substance P, in the ventrolateral and arcuate nuclei of the female guinea pig. Neurosci Res. 1999,33:223-8.

Dungan, H. M., Clifton, D. K., Steiner, R. A. Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. Endocrinology. 2006,147:1154-8.

Dutour, A., Briard, N., Guillaume, V., Magnan, E., Cataldi, M., Sauze, N., Oliver, C. Another view of GH neuroregulation: Lessons from the sheep. European Journal Of Endocrinology. 1997,136:553-65.

Elmquist, J.K. Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. Physiol Behav. 2001, 74, 703–08.

Estrada, K. M., Clay, C. M., Pompolo, S., Smith, J. T., Clarke, I. J. Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J Neuroendocrinology. 2006, 18:806-9.

Estrada, K. M., Pompolo, S., Morris, M. J., Tilbrook, A. J., Clarke, I. J. Neuropeptide Y (NPY) delays the estrogen-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 Neuroendocrinology. 2003 15:1011-20.

Etgen, A. M., Morales, J. C. Somatosensory stimuli evoke norepinephrine release in the anterior ventromedial hypothalamus of sexually receptive female rats. J Neuroendocrinology. 2002,14:213-8.

Evans, N. P., Dahl, G. E., Mauger, D., Karsch, F. J. Estradiol induces both qualitative and quantitative changes in the pattern of gonadotropin-releasing hormone secretion during the presurge period in the ewe. Endocrinology. 1995,136:1603-9.

Evans, N. P., Dahl, G. E., Glover, B. H., Karsch, F. J. Central regulation of pulsatile gonadotropin-releasing hormone (GnRH) secretion by estradiol during the period leading up to the preovulatory GnRH surge in the ewe. Endocrinology. 1994,134:1806-11.

Evans, N. P., Dahl, G. E., Padmanabhan, V., Thrun, L. A., Karsch, F. J. Estradiol requirements for induction and maintenance of the gonadotropin-releasing hormone surge: implications for neuroendocrine processing of the estradiol signal. Endocrinology. 1997, 138:5408-14.

Fabre-Nys, C. Steroid control of monoamines in relation to sexual behaviour. Rev Reprod. 1998, 3**:**31-41.

Fabre-Nys, C., Blache, D., Hinton, M. R., Goode, J. A., Kendrick, K. M. Microdialysis measurement of neurochemical changes in the mediobasal hypothalamus of ovariectomized ewes during oestrus. Brain Res. 1994, 649**:**282-96.

Fabre-Nys, C., Chesneau, D., de la Riva, C., Hinton, M. R., Locatelli, A., Ohkura, S., Kendrick, K. M. Biphasic role of dopamine on female sexual behaviour via D2 receptors in the mediobasal hypothalamus. Neuropharmacology. 2003,44:354-66.

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

Fabre-Nys, C., Martin, G. B. Hormonal control of proceptive and receptive sexual behavior and the preovulatory LH surge in the ewe: reassessment of the respective roles of estradiol, testosterone, and progesterone. Horm Behav. 1991, 25**:**295-312.

Fernandez-Fernandez R, Martini AC, Navarro VM, Castellano JM, Dieguez C, Aguilar E. Novel signals for the integration of energy balance and reproduction. Mol Cell Endocrinology. 2006,255:127–32.

Fabre-Nys, C., Ohkura, S., Kendrick, K. M. Male faces and odours evoke differential patterns of neurochemical release in the mediobasal hypothalamus of the ewe during oestrus: an insight into sexual motivation. Eur J Neurosci. 1997, 9:1666-77.

Fergani, C., Saifullizam A.K., Routly, J.E., Smith, R.F., Dobson, H. Oestrus behaviour, luteinising hormone and oestradiol profiles of intact ewes treated with insulin or endotoxin. Physiol Behav. 2011. Manuscript accepted for publication.

Fleischmann A, Makman MH, Etgen AM 1995 GABA-A receptor activation induces GABA and glutamate release from the preoptic area. Life Sci 56:1665–1678

Fletcher, I. C., Lindsay, D. R. Sensory involvement in the mating behaviour of domestic sheep. Anim Behav. 1968, 16**:**410-4.

Foradori, C. D., Amstalden, M., Coolen, L. M., Singh, S. R., McManus, C. J., Handa, R. J., Goodman, R. L., Lehman, M. N. Orphanin FQ: evidence for a role in the control of the reproductive neuroendocrine system. Endocrinology. 2007,148:4993-5001.

Foradori, C. D., Coolen, L. M., Fitzgerald, M. E., Skinner, D. C., Goodman, R. L., Lehman, M. N. Colocalization of progesterone receptors in the parvicellular dynorphin neurons of the ovine preoptic area and hypothalamus. Endocrinology. 2002, 143:4366–4374**.**

Foradori, C. D., Goodman, R. L., Lehman, M. N. Distribution of preprodynorphin mRNA and dynorphin-a immunoreactivity in the sheep preoptic area and hypothalamus. Neuroscience. 2005,130:409-18.

Foradori, C. D., Amstalden, M., Goodman, R. L., Lehman, M. N. Colocalisation of dynorphin a and neurokinin B immunoreactivity in the arcuate nucleus and median eminence of the sheep. J Neuroendocrinology. 2006,18:534-41.

Franceschini, I., Lomet, D., Cateau, M., Delsol, G., Tillet, Y., Caraty, A. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. Neurosci Lett. 2006, 401:225-30.

Gabriel, S. M., Koenig, J. I., Washton, D. L. Estrogen stimulation of galanin gene expression and galanin-like immunoreactivity in the rat and its blockade by the estrogen antagonist keoxifene (LY156758). Regul Pept. 1993,45:407-19.

Ghuman, S. P., Morris, R., Spiller, D. G., Smith, R. F., Dobson, H. Integration between different hypothalamic nuclei involved in stress and GnRH secretion in the ewe. Reprod Domest Anim. 2010,45:1065-73.

Ghuman, S.P.S., Morris, R., Scherzer, J., Routly, J.E., Mobasheri, A., Smith, R.F.,

Dobson, H. Neuronal responses in the brainstem and hypothalamic nuclei following insulin treatment during the late follicular phase in the ewe. Reprod Dom Anim. 2011, 46:121–129.

Ghuman, S. P., Jones, D. N., Prabhakar, S., Smith, R. F., Dobson, H. GABA control of GnRH release from the ewe hypothalamus in vitro: sensitivity to oestradiol. Reprod Domest Anim. 2008,43:531-41.

Gonzalez, M. I., Baker, B. I., Wilson, C. A. Stimulatory effect of melanin-concentrating hormone on luteinising hormone release. Neuroendocrinology. 1997,66:254-62.

Gonzalez, M. I., Celis, M. E., Hole, D. R., Wilson, C. A. Interaction of oestradiol, alpha-melanotrophin and noradrenaline within the ventromedial nucleus in the control of female sexual behaviour. Neuroendocrinology. 1993,58:218-26.

Goodman, R. L., Jansen, H. T., Billings, H. J., Coolen, L. M., Lehman, M. N. Neural systems mediating seasonal breeding in the ewe. J Neuroendocrinology. 2010, 22:674–81.

Goodman, R. L., Parfitt, D. B., Evans, N. P., Dahl, G. E., Karsch, F. J. Endogenous opioid peptides control the amplitude and shape of gonadotropin-releasing hormone pulses in the ewe. Endocrinology. 1995,136:2412-20.

Goodman, R. L. Neural systems mediating the negative feedback actions of estradiol and progesterone in the ewe. Acta Neurobiol Exp (Wars). 1996, 56**:**727-41.

Goodman, R.L., Lehman, M.N., Smith, J.T., Coolen, L.M., de Oliveira, C.V., Jafarzadehshirazi, M.R., Pereira, A., Iqbal, J., Caraty, A., Ciofi, P., Clarke, I.J. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology. 2007, 148:5752–60.

Goodman, R. L. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. Endocrinology. 1978,102:142-50.

Goodman, R. L. Neural systems mediating the negative feedback actions of estradiol and

progesterone in the ewe. Acta neurobiologiae experimentalis. 1996,56:727-41.

Goodman, R. L., Bittman, E. L., Foster, D. L., Karsch, F. J. The endocrine basis of the synergistic suppression of luteinizing hormone by estradiol and progesterone. Endocrinology. 1981,109:1414-7.

Goodman, R. L., Coolen, L. M., Anderson, G. M., Hardy, S. L., Valent, M., Connors, J. M., Fitzgerald, M. E., Lehman, M. N. Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. Endocrinology. 2004,145:2959-67.

Goodman, R. L., Karsch, F. J. Pulsatile secretion of luteinizing hormone: differential suppression by ovarian steroids. Endocrinology. 1980,107:1286-90.

Goubillon, M. L., Caraty, A., Herbison, A. E. Evidence in favour of a direct input from the ventromedial nucleus to gonadotropin-releasing hormone neurones in the ewe: an anterograde tracing study. J Neuroendocrinology. 2002, 14:95-100.

Goubillon, M., Delaleu, B., Tillet, Y., Caraty, A., Herbison, A. E. Localization of estrogen-receptive neurons projecting to the GnRH neuron-containing rostral preoptic area of the ewe. Neuroendocrinology. 1999, 70:228-36.

Goubillon ML, Forsdike RA, Robinson JE, Ciofi P, Caraty A, Herbison AE 2000 Identification of neurokinin B-expressing neurons as a highly estrogenreceptive, sexually dimorphic cell group in the ovine arcuate nucleus. Endocrinology**.** 141:4218–4225

Grafstein-Dunn, E., Clifton, D. K., Steiner, R. A. Regulation of galanin and gonadotropin-releasing hormone gene expression in the hypothalamus and basal forebrain of the rat. Horm Behav. 1994,28:417-23.

Graham, L.H., Bolling, J., Miller, G., Pratt-Hawkes, N., Joseph, S. Enzyme-Immunoassay for the measurement of luteinizing hormone in the serum of African elephants (Loxodonta africana). Zoo Biol. 2002, 21:403–8.

Hunyady, B., Krempels, K., Harta, G., Mezey, E. Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. J Histochem Cytochem. 1996, 44:1353-62.

Harris, T. G., Battaglia, D. F., Brown, M. E., Brown, M. B., Carlson, N. E., Viguie, C., Williams, C. Y., Karsch, F. J. Prostaglandins mediate the endotoxin-induced suppression of pulsatile gonadotropin-releasing hormone and luteinizing hormone secretion in the ewe. Endocrinology. 2000,141:1050-8.

Harris, T. G., Dye, S., Robinson, J. E., Skinner, D. C., Evans, N. P. Progesterone can block transmission of the estradiol-induced signal for luteinizing hormone surge generation during a specific period of time immediately after activation of the gonadotropin-releasing hormone surge-generating system. Endocrinology. 1999, 140**:**827-34.

Harris, T. G., Robinson, J. E., Evans, N. P., Skinner, D. C., Herbison, A. E. Gonadotropin-releasing hormone messenger ribonucleic acid expression changes before the onset of the estradiol-induced luteinizing hormone surge in the ewe. Endocrinology. 1998, 139**:**57-64.

Henry, B. A., Tilbrook, A. J., Dunshea, F. R., Rao, A., Blache, D., Martin, G. B., Clarke, I. J. Long-term alterations in adiposity affect the expression of melanin-concentrating hormone and enkephalin but not proopiomelanocortin in the hypothalamus of ovariectomized ewes. Endocrinology. 2000, 141:1506-14.

Henry, B. A., Rao, A., Tilbrook, A. J., Clarke, I. J. Chronic food-restriction alters the expression of somatostatin and growth hormone-releasing hormone in the ovariectomised ewe. Journal of Endocrinology. 2001,170:R1-R5.

Herbison, A. E., Theodosis, D. T. Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience. 1992, 50:283-98.

Herbison, A. E. Neurochemical identity of neurones expressing oestrogen and androgen

receptors in sheep hypothalamus. Journal of reproduction and fertility. Supplement. 1995,49:271-83.

Herbison, A. E. Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrine reviews. 1998,19:302-30.

Herbison, A. E. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). Brain research reviews. 2008,57:277-87.

Herbison, A. E., Pape, J. R. New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Frontiers in Neuroendocrinology. 2001,22:292-308.

Herbison, A. E., Robinson, J. E., Skinner, D. C. Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone. Neuroendocrinology. 1993,57:751-9.

Hill, J. W., Elmquist, J. K., Elias, C. F. Hypothalamic pathways linking energy balance and reproduction. Am J Physiol Endocrinol Metab. 2008,294:E827-32.

Hoffman, G. E., Le, W. W., Franceschini, I., Caraty, A., Advis, J. P. Expression of Fos and in Vivo Median Eminence Release of LHRH Identifies an Active Role for Preoptic Area Kisspeptin Neurons in Synchronized Surges of LH and LHRH in the Ewe. Endocrinology. 2010, 152:214-222.

Hoffman, G. E., Smith, M. S., Verbalis, J. G. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Frontiers in neuroendocrinology. 1993,14:173-213.

Horton, R.J.E., Cummins, J.T., Clarke, I.J. Naloxone evokes large-amplitude GnRH pulses in luteal-phase ewes. J Repro Fert. 1987, 81:277-86.

Hrabovszky, E., Steinhauser, A., Barabas, K., Shughrue, P.J., Petersen, S.L., Merchenthaler, I., Liposits, Z. Estrogen receptor-beta immunoreactivity in

luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology. 2001, 142:3261–64.

Irwig, M.S., Fraley, G.S., Smith, J.T., Acohido, B.V., Popa, S.M., Cunningham, M.J., Gottsch, M.L., Clifton, D.K., Steiner, R.A. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology. 2004, 80:264–72.

Iqbal, J., Henry, B. A., Pompolo, S., Rao, A., Clarke, I. J. Long-term alteration in bodyweight and food restriction does not affect the gene expression of either preproorexin or prodynorphin in the sheep. Neuroscience. 2003,118:217-26.

Iwasa, T., Matsuzak, T., Murakami, M., Shimizu, F., Kuwahara, A., Yasui, T., Irahara, M. Decreased expression of kisspeptin mediates acute immune/inflammatory stress-induced suppression of gonadotrophin secretion in female rat. J. Endocrinol. Invest. 2008, 31:656-9.

Jansen, H. T., Cutter, C., Hardy, S., Lehman, M. N., Goodman, R. L. Seasonal Plasticity within the Gonadotropin-Releasing Hormone (GnRH) System of the Ewe: Changes in Identified GnRH Inputs and Glial Association. Endocrinology. 2003,144:3663-76.

Jansen, H. T., Hileman, S. M., Lubbers, L. S., Jackson, G. L., Lehman, M. N. A subset of estrogen receptor-containing neurons project to the median eminence in the ewe. J Neuroendocrinology. 1996, 8:921-7.

Jansen, H. T., Hileman, S. M., Lubbers, L. S., Kuehl, D. E., Jackson, G. L., Lehman, M. N. Identification and distribution of neuroendocrine gonadotropin-releasing hormone neurons in the ewe. Biology of Reproduction. 1997,56:655-62.

Jansen, H. T., West, C., Lehman, M. N., Padmanabhan, V. Ovarian estrogen receptor-beta (ERbeta) regulation: I. Changes in ERbeta messenger RNA expression prior to ovulation in the ewe. Biology of Reproduction. 2001,65:866-72.

Jasoni, C. L., Todman, M. G., Han, S. K., Herbison, A. E. Expression of mRNAs encoding receptors that mediate stress signals in gonadotropin-releasing hormone neurons of the mouse. Neuroendocrinology. 2005, 82:320-8.

Johnston, R.E., Bronson, F. Endocrine control of female mouse odors that elicit luteinizing hormone surges and attraction in males. Biology of Reproduction 1982, 27:1174-80.

Kalra, S. P. Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. Endocrine reviews. 1993,14:507-38.

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

Kalra, S. P., Fuentes, M., Fournier, A., Parker, S. L., Crowley, W. R. Involvement of the Y-1 receptor subtype in the regulation of luteinizing hormone secretion by neuropeptide Y in rats. Endocrinology. 1992,130:3323-30.

Kalra, P.S., McCann, S.M. The stimulatory effect on gonadotrophin release of implants of estradiol or progesterone in certain sites in the central nervous system. Neuroendocrinology. 1975, 19:289–302.

Kalra, P. S., Bonavera, J. J., Kalra, S. P. Central administration of antisense oligodeoxynucleotides to neuropeptide Y (NPY) mRNA reveals the critical role of newly synthesized NPY in regulation of LHRH release. Regul Pept. 1995,59:215-20.

Karsch, F. J., Battaglia, D. F., Breen, K. M., Debus, N., Harris, T. G. Mechanisms for ovarian cycle disruption by immune/inflammatory stress. Stress. 2002, 5**:**101-12.

Karsch, F. J., Bowen, J. M., Caraty, A., Evans, N. P., Moenter, S. M. Gonadotropin-releasing hormone requirements for ovulation. Biology of Reproduction. 1997,56:303-9.

Karsch, F. J., Cummins, J. T., Thomas, G. B., Clarke, I. J. Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing hormone in the ewe. Biology of

Reproduction. 1987,36:1207-18.

Karsch, F. J., Legan, S. J., Ryan, K. D., Foster, D. L. Importance of estradiol and progesterone in regulating LH secretion and estrous behavior during the sheep estrous cycle. Biology of Reproduction 1980, 23**:**404-13.

Karsch, F. J., Battaglia, D. F. Mechanisms for endotoxin-induced disruption of ovarian cyclicity: observations in sheep. Reprod Suppl. 2002, 59, 101-13.

Kasa-Vubu, J. Z., Dahl, G. E., Evans, N. P., Thrun, L. A., Moenter, S. M., Padmanabhan, V., Karsch, F. J. Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone. Endocrinology. 1992,131:208-12.

Kaske, M., Elmahdi, B., von Engelhardt, W., Sallmann, H. P. Insulin responsiveness of sheep, ponies, miniature pigs and camels: results of hyperinsulinemic clamps using porcine insulin.J Comp Physiol B. 2001, 171:549-56.

Kauffman, A. S., Park, J. H., McPhie-Lalmansingh, A. A., Gottsch, M. L., Bodo, C., Hohmann, J. G., Pavlova, M. N., Rohde, A. D., Clifton, D. K., Steiner, R. A., Rissman, E. F. The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. J Neurosci. 2007, 27:8826-35.

Kawaguchi, M., Funabashi, T., Kimura, F. Insulin-induced hypoglycemia does not impair the surge of luteinizing hormone secretion in the proestrous rat. Neuroscience letters. 1998,256:131-4.

Kawano, H., Daikoku, S. Somatostatin-containing neuron systems in the rat hypothalamus: retrograde tracing and immunohistochemical studies. J Comp Neurol. 1988,271:293-9.

Kendrick, K. M., Fabre-Nys, C., Blache, D., Goode, J. A., Broad, K. D. The role of oxytocin release in the mediobasal hypothalamus of the sheep in relation to female sexual receptivity. J Neuroendocrinology. 1993,5:13-21.

Khorram, O., Pau, K. Y. F., Spies, H. G. Bimodal Effects Of Neuropeptide-Y On

Hypothalamic Release Of Gonadotropin-Releasing Hormone In Conscious Rabbits. Neuroendocrinology. 1987,45:290-7.

Kinoshita, M., Tsukamura, H., Adachi, S., Matsui, H., Uenoyama, Y., Iwata, K., Yamada, S., Inoue, K., Ohtaki, T., Matsumoto, H., Maeda, K. Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology. 2005,146:4431-6.

Kinsey-Jones, J. S., Li, X. F., Knox, A. M., Wilkinson, E. S., Zhu, X. L., Chaudhary, A. A., Milligan, S. R., Lightman, S. L., O'Byrne, K. T. 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 Neuroendocrinology. 2009, 21:20-9.

Kow, L. M., Weesner, G. D., Pfaff, D. W. Alpha 1-adrenergic agonists act on the ventromedial hypothalamus to cause neuronal excitation and lordosis facilitation: electrophysiological and behavioral evidence. Brain research. 1992,588:237-45.

Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J. A., Smithies, O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proceedings of the National Academy of Sciences of the United States of America. 1998,95:15677-82.

Kurunczi, A., Hoyk, Z., Csakvari, E., Gyenes, A., Parducz, A. 17beta-Estradiol-induced remodeling of GABAergic axo-somatic synapses on estrogen receptor expressing neurons in the anteroventral periventricular nucleus of adult female rats. Neuroscience. 2009,158:553-7.

Landefeld, T. D., Suttie, J. M. Changes in messenger ribonucleic acid concentrations and plasma levels of growth hormone during the ovine estrous cycle and in response to exogenous estradiol. Endocrinology. 1989,125:1474-8.

Latronico, A. C. The neurokinin B pathway in human reproduction. Nature genetics. 2009,41:269-70.

Lehman, M. N., Robinson, J. E., Karsch, F. J., Silverman, A. J. Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. J Comp Neurol. 1986,244:19-35.

Lehman, M. N., Coolen, L. M., Goodman, R. L. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. Endocrinology. 2010 a, 151:3479-89.

Lehman, M. N., Ebling, F. J., Moenter, S. M., Karsch, F. J. Distribution of estrogen receptor-immunoreactive cells in the sheep brain. Endocrinology. 1993, 133:876-86.

Lehman, M. N., Karsch, F. J. Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and beta-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology. 1993,133:887-95.

Lehman, M. N., Merkley, C. M., Coolen, L. M., Goodman, R. L. Anatomy of the kisspeptin neural network in mammals. Brain Res. 2010b, 1364:90-102.

Lenox, R. H., Wray, H. L., Kant, G. J., Hawkins, T. D., Meyerhoff, J. L. Changes in brain levels of cyclic nucleotides and gamma-aminobutyric acid in barbiturate dependence and withdrawal. Eur J Pharmacol. 1979,55:159-69.

Leranth, C., MacLusky, N. J., Shanabrough, M., Naftolin, F. Immunohistochemical evidence for synaptic connections between pro-opiomelanocortin-immunoreactive axons and LH-RH neurons in the preoptic area of the rat. Brain Res. 1988,449:167-76.

Li, X. F., Bowe, J. E., Kinsey-Jones, J. S., Brain, S. D., Lightman, S. L., O'Byrne, K. T. Differential role of corticotrophin-releasing factor receptor types 1 and 2 in stress-induced suppression of pulsatile luteinising hormone secretion in the female rat. J Neuroendocrinology. 2006, 18:602–10.

Li, X. F., Bowe, J. E., Lightman, S. L., O'Byrne, K. T. Role of corticotropin-releasing factor receptor-2 in stress-induced suppression of pulsatile luteinizing hormone secretion in the rat. Endocrinology. 2005, 146:318–322.

Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., Smithies, O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proceedings of the National Academy of Sciences of the United States of America. 1993,90:11162-6.

Luine, V. N., Grattan, D. R., Selmanoff, M. Gonadal hormones alter hypothalamic GABA and glutamate levels. Brain research. 1997,747:165-8.

Luque, R.M., Kineman, R.D., Tena-Sempere, M. Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. Endocrinology. 2007, 148:4601–11.

Maeda, K., Ohkura, S., Uenoyama, Y., Wakabayashi, Y., Oka, Y., Tsukamura, H., Okamura, H. Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus. Brain research. 2010,1364:103-15.

Macfarlane, M. S., Breen, K. M., Sakurai, H., Adams, B. M., Adams, T. E. Effect of duration of infusion of stress-like concentrations of cortisol on follicular development and the preovulatory surge of LH in sheep. Anim Reprod Sci. 2000,63:167-75.

Malven, P.V. Role of endogenous opioids for regulation of the oestrus cycle in sheep and cattle. Reproduction in Domestic Animals. 1999, 30:183-7.

Malven, P. V., Haglof, S. A., Degroot, H. Effects Of Intracerebral Administration Of Neuropeptide-Y On Secretion Of Luteinizing-Hormone In Ovariectomized Sheep. Brain Research Bulletin. 1992,28:871-5.

Malven, P. V., Haglof, S. A., Jiang, H. Serum concentrations of luteinizing hormone, growth hormone, and prolactin in untreated and estradiol-treated ovariectomized ewes after immunoneutralization of hypothalamic neuropeptide Y. J Anim Sci.

1995,73:2105-12.

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

Matthews, S. G., Parrott, R. F., Sirinathsinghji, D. J. Isolation- and dehydration-induced changes in neuropeptide gene expression in the sheep hypothalamus. J Mol Endocrinol. 1993,11:181-9.

McDevitt, M. A., Glidewell-Kenney, C., Jimenez, M. A., Ahearn, P. C., Weiss, J., Jameson, J. L., Levine, J. E. New insights into the classical and non-classical actions of estrogen: evidence from estrogen receptor knock-out and knock-in mice. Molecular and cellular endocrinology. 2008,290:24-30.

McShane, T. M., May, T., Miner, J. L., Keisler, D. H. Central Actions Of Neuropeptide-Y May Provide a Neuromodulatory Link Between Nutrition and Reproduction. Biology of Reproduction. 1992,46:1151-7.

Medina, C. L., Nagatani, S., Darling, T. A., Bucholtz, D. C., Tsukamura, H., Maeda, K., Foster, D. L. Glucose availability modulates the timing of the luteinizing hormone surge in the ewe. J Neuroendocrinology. 1998, 10**:**785-92.

Melis, M. R., Argiolas, A. Dopamine and sexual behavior. Neurosci Biobehav Rev. 1995,19:19-38.

Merchenthaler, I., Setalo, G., Csontos, C., Petrusz, P., Flerko, B., Negro-Vilar, A. Combined retrograde tracing and immunocytochemical identification of luteinizing hormone-releasing hormone- and somatostatin-containing neurons projecting to the median eminence of the rat. Endocrinology. 1989,125:2812-21.

Merkley, C., Coolen, L. M., Jackson, L., Goodman, R. L., Lehman, M. N. Evidence for transcriptional activation of arcuate kisspeptin neurons, and glutamatergic input to kisspeptin during the preovulatory GnRH surge of the sheep. Program of the 91st Annual Meeting of The Endocrine Society, Washington, DC, 2009 (Abstract P3-220)

Messager, S., Chatzidaki, E. E., Ma, D., Hendrick, A. G., Zahn, D., Dixon, J., Thresher, R.

R., Malinge, I., Lomet, D., Carlton, M. B., Colledge, W. H., Caraty, A., Aparicio, S. A. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proceedings of the National Academy of Sciences of the United States of America. 2005,102:1761-6.

Michael, R. P., Clancy, A. N., Zumpe, D. Mating activates estrogen receptor-containing neurons in the female monkey brain. Physiol Behav. 2005,85:404-13.

Moenter, S. M., Caraty, A., Karsch, F. J. The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. Endocrinology. 1990, 127**:**1375-84.

Moenter, S. M., Caraty, A., Locatelli, A., Karsch, F. J. Pattern of gonadotropin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. Endocrinology. 1991, 129:1175-82.

Moenter, S. M., DeFazio, R. A. Endogenous {gamma}-Aminobutyric Acid Can Excite Gonadotropin-Releasing Hormone Neurons. Endocrinology. 2005,146:5374-9.

Mogil, J. S., Pasternak, G. W. The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. Pharmacol Rev. 2001,53:381-415.

Mountjoy, P. D., Rutter, G. A. Glucose sensing by hypothalamic neurones and pancreatic islet cells: AMPle evidence for common mechanisms? Exp Physiol. 2007,92:311-9.

Munro, C., Stabenfeldt, G. Development of a microtitre plate enzyme immunoassay for the determination of progesterone. J Endocrinol. 1984, 101:41-49.

Navarro, V. M., Castellano, J. M., Fernandez-Fernandez, R., Tovar, S., Roa, J., Mayen, A., Nogueiras, R., Vazquez, M. J., Barreiro, M. L., Magni, P., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology. 2005,146:156-63.

Naylor, A.M., Porter, D.W., Lincoln, D.W. Central administration of corticotrophin-releasing factor in the sheep: effects on secretion of gonadotrophins, prolactin and cortisol. J.Endocrinol. 1990, 124:117–125.

Neal, C. R., Jr., Mansour, A., Reinscheid, R., Nothacker, H. P., Civelli, O., Watson, S. J., Jr. Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. The Journal of comparative neurology. 1999,406:503-47.

Noel, S. D., Keen, K. L., Baumann, D. I., Filardo, E. J., Terasawa, E. Involvement of G protein-coupled receptor 30 (GPR30) in rapid action of estrogen in primate LHRH neurons. Molecular endocrinology (Baltimore, Md.). 2009,23:349-59.

Oakley, A. E., Breen, K. M., Tilbrook, A. J., Wagenmaker, E. R., Karsch, F. J. Role of estradiol in cortisol-induced reduction of luteinizing hormone pulse frequency. Endocrinology. 2009, 150**:**2775-82.

Oakley, A. E., Clifton, D. K., Steiner, R. A. Kisspeptin signaling in the brain. Endocr Rev. 2009, 30:713-43.

Ohkura, S., Tanaka, T., Nagatani, S., Bucholtz, D. C., Tsukamura, H., Maeda, K., Foster, D. L. Central, but not peripheral, glucose-sensing mechanisms mediate glucoprivic suppression of pulsatile luteinizing hormone secretion in the sheep. Endocrinology. 2000,141:4472-80.

Okamura, H., Murata, K., Sakamoto, K., Wakabayashi, Y., Ohkura, S., Takeuchi Y., Mori, Y. Male Effect Pheromone Tickles the Gonadotrophin-Releasing Hormone Pulse Generator. J Neuroendocrinology. 2010, 22:825–832.

Olster, D. H., Blaustein, J. D. Development of progesterone-facilitated lordosis in female guinea pigs: relationship to neural estrogen and progestin receptors. Brain research. 1989,484:168-76.

Orikasa, C., Sakuma, Y. Possible involvement of preoptic estrogen receptor beta positive cells in luteinizing hormone surge in the rat. Domestic animal endocrinology.

2003,25:83-92.

Orikasa, C., Sakuma, Y. Possible involvement of preoptic estrogen receptor beta positive cells in luteinizing hormone surge in the rat. Domest Anim Endocrinol. 2003,25:83-92.

Papargiris, M.M., Rivalland, E.T., Hemsworth, P.H., Morrissey, A.D., Tilbrook, A.J. Acute and chronic stress-like levels of cortisol inhibit the oestradiol stimulus to induce sexual receptivity but have no effect on sexual attractivity or proceptivity in female sheep. Horm Behav. 2011

Pawson, A. J., Morgan, K., Maudsley, S. R., Millar, R. P. Type II gonadotrophin-releasing hormone (GnRH-II) in reproductive biology. Reproduction. 2003, 126:271-8.

Pelletier, G. Ultrastructural localization of neuropeptide Y in the hypothalamus. Ann N Y Acad Sci. 1990,611:232-46.

Penicaud, L., Leloup, C., Fioramonti, X., Lorsignol, A., Benani, A. Brain glucose sensing: a subtle mechanism. Curr Opin Clin Nutr Metab Care. 2006,9:458-62.

Pereira, A., Rawson, J., Jakubowska, A., Clarke, I.J. Estradiol-17β-responsive A1 and A2 noradrenergic cells of the brain stem project to the bed nucleus of the stria terminalis in the ewe brain: a possible route for the regulation of the gonadotrophin releasing hormone cells. Neuroscience. 2010, 165:758–773.

Petersen, S.L., Barraclough, C.A. Suppression of spontaneous LH surges in estrogen-treated ovariectomized rats by microimplants of antiestrogens into the preoptic brain. Brain Res. 1989, 484:279–89

Petersen, S. L., Keller, M. L., Carder, S. A., McCrone, S. Differential-Effects Of Estrogen and Progesterone On Levels Of POMC Messenger-RNA Levels In the Arcuate Nucleus - Relationship to the Timing Of LH Surge Release. J Neuroendocrinology. 1993,5:643-8.

Phogat, J. B., Smith, R. F., Dobson, H. Effect of transport on pituitary responsiveness to exogenous pulsatile GnRH and oestradiol-induced LH release in intact ewes. J

Reprod Fertil. 1999, 116:9-18.

Pierce, A. A., Iwueke, I., Ferkin, M. H. Food deprivation and the role of estradiol in mediating sexual behaviors in meadow voles. Physiol Behav. 2007, 90:353-61.

Pierce, B. N., Hemsworth, P. H., Rivalland, E. T., Wagenmaker, E. R., Morrissey, A. D., Papargiris, M. M., Clarke, I. J., Karsch, F. J., Turner, A. I., Tilbrook, A. J. Psychosocial stress suppresses attractivity, proceptivity and pulsatile LH secretion in the ewe. Horm Behav. 2008, 54:424-34.

Pierce, B. N., Stackpole, C. A., Breen, K. M., Clarke, I. J., Karsch, F. J., Rivalland, E. T., Turner, A. I., Caddy, D. J., Wagenmaker, E. R., Oakley, A. E., Tilbrook, A. J. Estradiol enables cortisol to act directly upon the pituitary to suppress pituitary responsiveness to GnRH in sheep. Neuroendocrinology. 2009, 89:86-97.

Pillon, D., Caraty, A., Fabre-Nys, C., Bruneau, G. Early decrease of proopiomelanocortin but not neuropeptide Y mRNA expression in the mediobasal hypothalamus of the ewe, during the estradiol-induced preovulatory LH surge. General and comparative endocrinology. 2003,134:264-72.

Pillon, D., Caraty, A., Fabre-Nys, C., Bruneau, G. Short-term effect of oestradiol on neurokinin B mRNA expression in the infundibular nucleus of ewes. J Neuroendocrinology. 2003,15:749-53.

Pillon, D., Carary, A., Fabre-Nys, C., Lomet, D., Cateau, M., Bruneau, G. Regulation by estradiol of hypothalamic somatostatin gene expression: possible involvement of somatostatin in the control of luteinizing hormone secretion in the ewe. Biology of Reproduction. 2004, 71:38–44.

Ping, L., Mahesh. V. B., Brann, D. W. Release of glutamate and aspartate from the preoptic area during the progesterone-induced LH surge: *in vivo* microdialysis studies. Neuroendocrinology. 1994, 59:318–24.

Plant, T. M., Ramaswamy, S., Dipietro, M. J. Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile

monkey (Macaca mulatta) elicits a sustained train of gonadotropin-releasing hormone discharges. Endocrinology. 2006,147:1007-13.

Polkowska, J. Stress and nutritional influences on GnRH and somatostatin neuronal systems in the ewe. Acta Neurobiol Exp (Wars). 1996,56:797-806.

Polkowska, J., Gladysz, A. Effect of food manipulation on the neuropeptide Y neuronal system in the diencephalon of ewes. J Chem Neuroanat. 2001,21:149-59.

Pompolo, S., Pereira, A., Estrada, K. M., Clarke, I. J. Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology. 2006, 147:804-10.

Pompolo, S., Rawson, J. A., Clarke, I. J. Projections from the arcuate/ventromedial region of the hypothalamus to the preoptic area and bed nucleus of stria terminalis in the brain of the ewe; lack of direct input to gonadotropin-releasing hormone neurons. Brain Res. 2001, 904:1-12.

Pompolo, S., Pereira, A., Kaneko, T., Clarke, I. J. Seasonal changes in the inputs to gonadotropin-releasing hormone neurones in the ewe brain: an assessment by conventional fluorescence and confocal microscopy. J Neuroendocrinology. 2003a,15:538-45.

Pompolo, S., Pereira, A., Scott, C. J., Fujiyma, F., Clarke, I. J. Evidence for estrogenic regulation of gonadotropin-releasing hormone neurons by glutamatergic neurons in the ewe brain: An immunohistochemical study using an antibody against vesicular glutamate transporter-2. The Journal of comparative neurology. 2003b,465:136-44.

Porter, D. W., Naylor, A. M., McNeilly, A. S., Lincoln, D. W. Endocrine actions of central neuropeptide Y in the ewe: activation of the hypothalamo-pituitary-adrenal axis by exogenous neuropeptide Y and role of endogenous neuropeptide Y in the secretion of luteinizing hormone during the oestrous cycle. J Neuroendocrinology. 1993,5:163-74.

Qi, Y., Iqbal, J., Oldfield, B. J., Clarke, I. J. Neural connectivity in the mediobasal hypothalamus of the sheep brain. Neuroendocrinology. 2008, 87:91-112.

Ramaswamy, S., Seminara, S. B., Ali, B., Ciofi, P., Amin, N. A., Plant, T. M. Neurokinin B stimulates GnRH release in the male monkey (Macaca mulatta) and is colocalized with kisspeptin in the arcuate nucleus. Endocrinology. 2010,151:4494-503.

Rawson, J. A., Scott, C. J., Pereira, A., Jakubowska, A., Clarke, I. J. Noradrenergic projections from the A1 field to the preoptic area in the brain of the ewe and Fos responses to oestrogen in the A1 cells. J Neuroendocrinology. 2001,13:129-38.

Rettori, V., Dees, W. L., Hiney, J. K., Lyson, K., McCann, S. M. An interleukin-1-alpha-like neuronal system in the preoptic-hypothalamic region and its induction by bacterial lipopolysaccharide in concentrations which alter pituitary hormone release. Neuroimmunomodulation. 1994,1:251-8.

Richter, T.A., Robinson, J.E., Evans, N.P. Progesterone blocks the estradiol-stimulated luteinizing hormone surge by disrupting activation in response to a stimulatory estradiol signal in the ewe. Biology of Reproduction. 2002, 67, 119–25.

Richter, T. A., Robinson, J. E., Lozano, J. M., Evans, N. P. Progesterone can block the preovulatory gonadotropin-releasing hormone/luteinising hormone surge in the ewe by a direct inhibitory action on oestradiol-responsive cells within the hypothalamus. J Neuroendocrinology. 2005, 17:161-9.

Rissman, E. F., Early, A. H., Taylor, J. A., Korach, K. S., Lubahn, D. B. Estrogen receptors are essential for female sexual receptivity. Endocrinology. 1997,138:507-10.

Rivalland, E. T. A., Iqbal. J., Clarke, I. J., Turner, A. I., Tilbrook, A. J. Co-localization and distribution of corticotrophin-releasing hormone, arginine vasopressin and enkephalin in the paraventricular nucleus of sheep: a sex comparison. Neuroscience. 2005, 132:755-66.

Rivier, C., Rivest, S. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biology of Reproduction. 1991,45:523-32.

Roa, J.,Vigo, E.,Castellano, J.M.,Navarro,V.M., Fernandez-Fernandez, R., Casanueva, F.F., Dieguez, C., Aguilar, E., Pinilla, L., Tena-Sempere, M. Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female rat. Endocrinology. 2006, 147:2864–78.

Robinson, J.E., Grindrod, J., Jeurissen, S., Taylor, J.A., Unsworth, W.P. Prenatal exposure of the ovine fetus to androgens reduces the proportion of neurons in the ventromedial and arcuate nucleus that are activated by short-term exposure to estrogen. Biology of Reproduction. 2010, 82:163–170

Robinson, J. E., Kendrick, K. M. Inhibition of luteinizing hormone secretion in the ewe by progesterone: associated changes in the release of gamma aminobutyric acid and noradrenaline in the preoptic area measured by intracranial microdialysis. J Neuroendocrinology. 1992, **4**:231-36.

Robinson, J. E., Kendrick, K. M., Lambart, C. E. Changes in the release of gamma-aminobutyric Acid and catecholamines in the preoptic/septal area prior to and during the preovulatory surge of luteinizing hormone in the ewe. J Neuroendocrinology. 1991,3:393-9.

Ronnekleiv, O.K., Kelly, M.J. Plasma prolactin and luteinizing hormone profiles during the estrous cycle of the female rat: effects of surgically induced persistent estrus. Neuroendocrinology. 1998, 47:133–141.

Roseweir, A. K., Millar, R. P. The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update. 2009, 15:203-12.

Rosie, R., Thomson, E., Blum, M., Roberts, J. L., Fink, G. Estrogen Positive Feedback Reduces Arcuate Proopiomelanocortin Messenger-Ribonucleic-Acid. J Neuroendocrinology. 1992,4:625-30.

Rotsztejn, W. H., Drouva, S. V., Epelbaum, J., Kordon, C. Somatostatin inhibits in vitro release of luteinizing hormone releasing hormone from rat mediobasal hypothalamic slices. Experientia. 1982,38:974-5.

Routh, V.H. Glucosensing neurons in the ventromedial hypothalamic nucleus (VMN) and hypoglycemia-associated autonomic failure (HAAF). Diabetes Metab Res Rev. 2003, 19:348–56.

Rubin, B. S., Barfield, R. J. Priming of estrous responsiveness by implants of 17 beta-estradiol in the ventromedial hypothalamic nucleus of female rats. Endocrinology. 1980,106:504-9.

Sahu, A., Crowley, W. R., Kalra, S. P. Evidence That Hypothalamic Neuropeptide-Y Gene-Expression Increases Before the Onset Of the Preovulatory LH Surge. J Neuroendocrinology. 1995,7:291-6.

Saifullizam A.K., Routly, J.E., Smith, R.F., Dobson, H. Effect of insulin on the relationship of estrous behaviors to estradiol and LH surges in intact ewes. Physiol and Behav. 2010, 99:555-61.

Scanlan, N., Dufourny, L., Skinner, D. C. Somatostatin-14 neurons in the ovine hypothalamus: colocalization with estrogen receptor alpha and somatostatin-28 (1–12) immunoreactivity, and activation in response to estradiol. Biology of Reproduction. 2003; 69:1318–1324.

Scanlan, N., Dufourny, L., Skinner, D. C. Somatostatin-14 neurons in the ovine hypothalamus: colocalization with estrogen receptor alpha and somatostatin-28(1-12) immunoreactivity, and activation in response to estradiol. Biology of Reproduction. 2003,69:1318-24.

Scanlan, N., Skinner, D. C. Estradiol Modulation of Growth Hormone Secretion in the Ewe: No Growth Hormone-Releasing Hormone Neurons and Few Somatotropes Express Estradiol Receptor {alpha}. Biology of Reproduction 2002,66:1267-73.

Scaramuzzi, R. J., Tillson, S. A., Thorneycroft, I. H., Caldwell, B. V. Action of exogenous progesterone and estrogen on behavioral estrus and luteinizing hormone levels in the ovariectomized ewe. Endocrinology. 1971, 88**:**1184-9.

Schioth, H. B., Watanobe, H. Melanocortins and reproduction. Brain Res Brain Res Rev.

2002,38:340-50.

Scimonelli, T., Medina, F., Wilson, C., Celis, M. E. Interaction of alpha-melanotropin (alpha-MSH) and noradrenaline in the median eminence in the control of female sexual behavior. Peptides. 2000,21:219-23.

Scott, C. J., Tilbrook, A. J., Simmons, D. M., Rawson, J. A., Chu, S., Fuller, P. J., Ing, N. H., Clarke, I. J. The distribution of cells containing estrogen receptor-alpha (ERalpha) and ERbeta messenger ribonucleic acid in the preoptic area and hypothalamus of the sheep: comparison of males and females. Endocrinology. 2000,141:2951-62.

Sejian, V., Maurya, V.P., Naqvi, S.M., Kumar, D., Joshi, A. Effect of induced body condition score differences on physiological response, productive and reproductive performance of Malpura ewes kept in a hot, semi-arid environment. J Anim Physiol Anim Nutr (Berl). 2010, 94:154-61.

Seminara, S. B. Metastin and its G protein-coupled receptor, GPR54: critical pathway modulating GnRH secretion. Frontiers in neuroendocrinology. 2005,26:131-8.

Shakil, T., Snell, A., Whitehead, S. A. Effects of lipopolysaccharide and cyclosporin on the endocrine control of ovarian function. J Reprod Fertil. 1994, 100**:**57-64.

Shivers, B. D., Harlan, R. E., Morrell, J. I., Pfaff, D. W. Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature. 1983, 304: 345-7.

Simonian, S. X., Delaleu, B., Caraty, A., Herbison, A. E. Estrogen receptor expression in brainstem noradrenergic neurons of the sheep. Neuroendocrinology. 1998,67:392-402.

Sinchak, K., Hendricks, D. G., Baroudi, R., Micevych, P. E. Orphanin FQ/nociceptin in the ventromedial nucleus facilitates lordosis in female rats. Neuroreport. 1997,8:3857-60.

Skinner, D. C., Caraty, A., Allingham, R. Unmasking the progesterone receptor in the preoptic area and hypothalamus of the ewe: no colocalization with gonadotropin-releasing neurons. Endocrinology. 2001, 142:573-9.

Skinner, D. C., Dufourny, L. Oestrogen receptor beta-immunoreactive neurones in the ovine hypothalamus: distribution and colocalisation with gonadotropin-releasing hormone. J Neuroendocrinology. 2005,17:29-39.

Skinner, D. C., Evans, N. P., Delaleu, B., Goodman, R. L., Bouchard, P., Caraty, A. The negative feedback actions of progesterone on gonadotropin-releasing hormone secretion are transduced by the classical progesterone receptor. Proceedings of the National Academy of Sciences of the United States of America. 1998,95:10978-83.

Skinner, D. C., Herbison, A. E. Effects of photoperiod on estrogen receptor, tyrosine hydroxylase, neuropeptide Y, and beta-endorphin immunoreactivity in the ewe hypothalamus. Endocrinology. 1997,138:2585-95.

Skinner, D. C., Malpaux, B., Delaleu, B., Caraty, A. Luteinizing hormone (LH)-releasing hormone in third ventricular cerebrospinal fluid of the ewe: correlation with LH pulses and the LH surge. Endocrinology. 1995,136:3230-7.

Skynner, M. J., Sim, J. A., Herbison, A. E. Detection of estrogen receptor alpha and beta messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons. Endocrinology. 1999,140:5195-201.

Sliwowska, J. H., Billings, H. J., Goodman, R. L., Lehman, M. N. Immunocytochemical colocalization of GABA-B receptor subunits in gonadotropin-releasing hormone neurons of the sheep. Neuroscience. 2006,141:311-9.

Smith, R. F., Dobson, H. Hormonal interactions within the hypothalamus and pituitary with respect to stress and reproduction in sheep. Domest Anim Endocrinol. 2002, 23, 75-85.

Smith, R. F., Ghuman, S. P., Evans, N. P., Karsch, F. J., Dobson, H. Stress and the control

of LH secretion in the ewe. Reprod Suppl. 2003, 61**:**267-82.

Smith, J. T. Kisspeptin signalling in the brain: steroid regulation in the rodent and ewe. Brain Res Rev. 2008, 57:288-98.

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

Smith, J. T., Clay, C. M., Caraty, A., Clarke, I. J. KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. Endocrinology. 2007, 148:1150-7.

Smith, J.T., Dungan, H.M., Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., Clifton, D.K., Steiner, R.A. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology. 2005, 146:2976–84

Smith, M. J., Jennes, L. Neural signals that regulate GnRH neurones directly during the oestrous cycle. Reproduction. 2001,122:1-10.

Smith, J. T., Li, Q., Pereira, A., Clarke, I. J. Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology. 2009, 150:5530–38.

Smith, J. T., Li, Q., Yap, K. S., Shahab, M., Roseweir, A. K., Millar, R. P., Clarke, I. J. Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence. Endocrinology. 2011, 152:1001-12.

Smith, J.T., Popa, S.M., Clifton, D.K., Hoffman, G.E., Steiner, R.A. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. Journal of Neuroscience. 2006, 26:6687– 94.

Smith, R. F., Ghuman, S. P., Evans, N. P., Karsch, F. J., Dobson, H. Stress and the control of LH secretion in the ewe. Reprod Suppl. 2003, 61:267-82.

Smith, J. T., Coolen, L. M., Kriegsfeld, L. J., Sari, I. P., Jaafarzadehshirazi, M. R., Maltby,

M., Bateman, K., Goodman, R. L., Tilbrook, A. J., Ubuka, T., Bentley, G. E., Clarke, I. J., Lehman, M. N. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology. 2008,149:5770-82.

Spiteri, T., Musatov, S., Ogawa, S., Ribeiro, A., Pfaff, D. W., Agmo, A. Estrogen-induced sexual incentive motivation, proceptivity and receptivity depend on a functional estrogen receptor alpha in the ventromedial nucleus of the hypothalamus but not in the amygdala. Neuroendocrinology. 2010,91:142-54.

Sullivan, K. A., Witkin, J. W., Ferin, M., Silverman, A. J. Gonadotropin-releasing hormone neurons in the rhesus macaque are not immunoreactive for the estrogen receptor. Brain research. 1995,685:198-200.

Sun, J., Chu, Z., Moenter, S. M. Diurnal in vivo and rapid in vitro effects of estradiol on voltage-gated calcium channels in gonadotropin-releasing hormone neurons. The Journal of neuroscience. 2010,30:3912-23.

Suzuki, C., Yoshioka, K., Iwamura, S., Hirose, H. Endotoxin induces delayed ovulation following endocrine aberration during the proestrous phase in Holstein heifers. Domest Anim Endocrinol. 2001, 20**:**267-78.

Taylor, J.A., Goubillon, M.L., Broad, K.D., Robinson, J.E. Steroid control of gonadotropin-releasing hormone secretion: associated changes in pro-opiomelanocortin and preproenkephalin messenger RNA expression in the ovine hypothalamus. Biology of Reproduction. 2007,76:524-531.

Tellam, D. J., Mohammad, Y. N., Lovejoy, D. A. Molecular integration of hypothalamo-pituitary-adrenal axis-related neurohormones on the GnRH neuron. Biochem Cell Biol. 2000,78:205-16.

Tena-Sempere M. Roles of ghrelin and leptin in the control of reproductive function. Neuroendocrinology 2007;86: 229–41.

Terry, L. C., Willoughby, J. O., Braseau, P., Martin, J. B., Patel, Y. Antiserum to somatostatin prevents stress-induced inhibition of growth hormone secretion in the rat. Science. 1976,192:565-7.

Thind, K. K., Goldsmith, P. C. Infundibular gonadotropin-releasing hormone neurons are inhibited by direct opioid and autoregulatory synapses in juvenile monkeys. Neuroendocrinology. 1988,47:203-16.

Thind, K. K., Goldsmith, P. C. Expression of estrogen and progesterone receptors in glutamate and GABA neurons of the pubertal female monkey hypothalamus. Neuroendocrinology. 1997,65:314-24.

Tilbrook, A. J., Pearce, D. T. Time required for spermatozoa to remain in the vagina of the ewe to ensure conception. Aust J Biol Sci. 1986, 39**:**305-9.

Tilbrook, A. J., Turner, A. I., Clarke, I. J. Stress and reproduction: central mechanisms and sex differences in non-rodent species. Stress. 2002, 5:83-100.

Tillet, Y., Caldani, M., Batailler, M. Anatomical relationships of monoaminergic and Neuroprptide Y-containing fibres with luteinizing hormone-releasing hormone systems in the preoptic area of the sheep brain: immunohistochemical studies. Journal of Chemical Neuroanatomy. 1989, 2:319-326.

Tillet, Y., Thibault, J. Catecholamine-containing neurons in the sheep brainstem and diencephalon: immunohistochemical study with tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH) antibodies. J Comp Neurol. 1989,290:69-104.

Tomaszewska-Zaremba, D., Mateusiak, K., Przekop, F. The role of GabaA receptors in the neural systems of the ventromedial hypothalamus-nucleus infundibular region in the control of GnRH release in ewes during follicular phase. Exp Clin Endocrinol Diabetes. 2003,111:335-40.

Topaloglu, A. K., Reimann, F., Guclu, M., Yalin, A. S., Kotan, L. D., Porter, K. M., Serin, A., Mungan, N. O., Cook, J. R., Ozbek, M. N., Imamoglu, S., Akalin, N. S., Yuksel, B., O'Rahilly, S., Semple, R. K. TAC3 and TACR3 mutations in familial

hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nature genetics. 2009,41:354-8.

Tourlet, S., Ziyazetdinova, G., Caraty, A., Tramu, G., Delsol, G., Tillet, Y. Oestradiol effect on galanin-immunoreactive neurones in the diencephalon of the ewe. J Neuroendocrinology. 2005,17:145-51.

van Pett, K., Viau, V., Bittencourt, J. C., Chan, R. K., Li, H. Y., Arias, C., Prins, G. S, Perrin, M., Vale, W., Sawchenko, P. E. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol. 2000, 428:191–212.

Van Vugt, H. H., Swarts, J. J. M., Van de Heijning, B. J. M., Van der Beek, E. M. Centrally applied somatostatin inhibits the luteinizing hormone surge in female rats. In: Program of the 33rd annual meeting of Society of Neuroscience; 2003; New Orleans, LA. Abstract 237.5.

Vellucci, S.V., Parrott, R.F. Expression of c-fos in the ovine brain following different types of stress, or central administration of corticotrophin-releasing hormone. Exp Physiol. 1994, 79:241-48.

Wahab, F., Ullah, F., Chan, Y. M., Seminara, S. B., Shahab, M. Decrease in hypothalamic Kiss1 and Kiss1r expression: a potential mechanism for fasting-induced suppression of the HPG axis in the adult male rhesus monkey (Macaca mulatta). Horm Metab Res. 2011, 43:81-5.

Wagenmaker, E. R., Breen, K. M., Oakley, A. E., Pierce, B. N., Tilbrook, A. J., Turner, A. I., Karsch, F. J. Cortisol interferes with the estradiol-induced surge of luteinizing hormone in the ewe. Biology of reproduction. 2009,80:458-63.

Wagenmaker, E. R., Breen, K. M., Oakley, A. E., Tilbrook, A. J., Karsch, F. J. Psychosocial stress inhibits amplitude of gonadotropin-releasing hormone pulses independent of cortisol action on the type II glucocorticoid receptor. Endocrinology. 2009,150:762-9.

Walker, S. L., Smith, R. F., Jones, D. N., Routly, J. E., Dobson, H. Chronic stress, hormone profiles and estrus intensity in dairy cattle. Horm Behav. 2008, 53:493–501.

Walsh, J.P., Clarke, I.J. Effects of central administration of highly selective opioid μ, δ and κ-receptor agonists on plasma luteinizing hormone (LH), prolactin, and the estrogen-induced LH surge in ovariectomized ewes. Endocrinology. 1996, 137:3640-48.

Walsh, J. P., Rao, A., Simmons, D. M., Clarke, I. J. Proopiomelanocortin mRNA levels in ovine hypothalamus are not reduced at the time of the preovulatory luteinizing hormone surge. J Neuroendocrinology. 1998, 10:803-808.

Walsh, J. P., Rao, A., Thompson, R. C., Clarke, I. J. Proenkephalin and opioid mu-receptor mRNA expression in ovine hypothalamus across the estrus cycle. J Endocrinology. 2001, 73:26-36.

Wankowska, M., Lerrant, Y., Wojcik-Gladysz, A., Starzec, A., Counis, R., Polkowska, J. Intracerebroventricular infusion of neuropeptide Y up-regulates synthesis and accumulation of luteinizing hormone but not follicle stimulating hormone in the pituitary cells of prepubertal female lambs. J Chem Neuroanat. 2002,23:133-42.

Watanobe, H., Takebe, K. Evidence That Neuropeptide-Y Secretion In the Median-Eminence Increases Prior to the Luteinizing-Hormone Surge In Ovariectomized Steroid-Primed Rats - Estimation By Push-Pull Perfusion. Neuroscience Letters. 1992,146:57-9.

Watson, R.E. Jr, Wiegand S.J., Clough R.W., Hoffman G.E. Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides. 7, 155-9.

Welento, J., Szteyn, S., Milart, Z. Observations on the stereotaxic configuration of the hypothalamus nuclei in the sheep. Anat. Anz. 1969, 124:1–27.

Werner, H., Koch, Y., Baldino, F., Jr., Gozes, I. Steroid regulation of somatostatin mRNA in the rat hypothalamus. J Biol Chem. 1988,263:7666-71.

Whisnant, C. S., Curto, K., Goodman, R. L. Immunocytochemical localization of beta endorphin and gonadal steroid regulation of proopiomelanocortin messenger ribonucleic acid in the ewe. Neuroendocrinology. 1992,56:812-21.

Willoughby, J. O., Oliver, J. R., Fletcher, T. P., Clarke, I. J. Distribution of Somatostatin Immunoreactivity in Sheep Hypothalamus - a Comparison With That of the Rat. Archives of Histology and Cytology. 1995,58:31-6.

Williams, C. Y., Harris, T. G., Battaglia, D. F., Viguie, C., Karsch, F. J. Endotoxin inhibits pituitary responsiveness to gonadotropin-releasing hormone. Endocrinology. 2001, 142:1915-22.

Wise, P. M., Scarbrough, K., Weiland, N. G., Larson, G. H. Diurnal pattern of proopiomelanocortin gene expression in the arcuate nucleus of proestrous, ovariectomized, and steroid-treated rats: a possible role in cyclic luteinizing hormone secretion. Mol Endocrinol. 1990,4:886-92.

Wisnant, C. S., Curtom K., Goodman, R. L. Immunocytochemical localization of beta endorphin and gonadal steroid regulation of proopiomelanocortin messenger ribonucleic acid in the ewe. J Neuroendocrinology. 1992, 56**:**812-821.

Wisnant, C. S., Goodman, R. L. Effects of an opioid antagonist on pulsatile luteinizing hormone secretion in the ewe vary with changes in steroid negative feedback. Biology of Reproduction. 1988, 39**:**1032-38.

Woller, M. J., McDonald, J. K., Reboussin, D. M., Terasawa, E. Neuropeptide Y is a neuromodulator of pulsatile luteinizing hormone-releasing hormone release in the gonadectomized rhesus monkey. Endocrinology. 1992,130:2333-42.

Woller, M. J., Terasawa, E. Estradiol enhances the action of neuropeptide Y on in vivo luteinizing hormone-releasing hormone release in the ovariectomized rhesus monkey. Neuroendocrinology. 1992,56:921-5.

Xia, L., Van Vugt, D., Alston, E. J., Luckhaus, J., Ferin, M. A surge of gonadotropin-releasing hormone accompanies the estradiol-induced gonadotropin surge in the

rhesus monkey. Endocrinology. 1992,131:2812-20.

Yang, K., Haynes, N. B., Lamming, G. E., Brooks, A. N. Ovarian steroid involvement in endogenous opioid modulation of LH secretion in mature ewes during the breeding and non-breeding seasons. Journal of Reproduction and Fertility. 1988, 83:129-39.

Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Monfort, S.L., Brown, J.L. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticold analyses. Gen and Comp Endo. 2004, 137:148-165.

Zorrilla, R., Simard, J., Rheaume, E., Labrie, F., Pelletier, G. Multihormonal control of pre-pro-somatostatin mRNA levels in the periventricular nucleus of the male and female rat hypothalamus. Neuroendocrinology. 1990,52:527-36.