NEUROENDOCRINOLOGY OF STRESS AND REPRODUCTION INTERACTION

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Sarvpreet Singh Ghuman

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Declaration

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

Sarvpreet Singh Ghuman

Contents

Abstract

Acknowledgements

List of publications/presentations

Abbreviations

Chapter 1 Introduction and literature review, 1

Introduction, 2

Distribution of GnRH neurones, 2

GnRH neurones related to tonic/surge release, 3

Steroid receptor distribution and identity of steroid receptor-positive neurones, 6

Neuronal projections to GnRH cell bodies and terminals, 11

Locus of steroid action and interaction in the brain to regulate GnRH secretion, 12

Neurotransmitters, 17

GnRH surge-induction process and its interruption, 33

Activation of stress pathways and regulation of GnRH, 37

Summary, 37

Objectives, 43

Chapter 2 General materials and methods, 44

In vitro perifusion procedures, 45

Media, 45

Hypothalamic slice and anterior pituitary collection, 45

Perifusion system, 46

Treatments, 47

Hypothalamic slice and anterior pituitary tissue viability, 48

Radioimmunoassay of hormones, 48

Arginine vasopressin, 48

Gonadotrophin releasing hormone, 49

Luteinising hormone, 49

Cortisol, 49

Plasma Glucose estimation, 50

Immunohistochemistry procedures, 50

Tissue collection, 50

Antisera, 51

Staining procedures, 51

Light microscopy, 52

Laser scanning confocal microscopy, 52

Chapter 3 Oestradiol stimulates the release AVP and GnRH from ewe hypothalamus *in vitro*, 55

Abstract, 56

Introduction, 56

Materials and methods, 58

Results, 59

Discussion, 60

Chapter 4 GABA control of arginine vasopressin release from ewe hypothalamus *in vitro*: sensitivity to oestradiol, 66

Abstract, 67

Introduction, 67

Materials and methods, 70

Results, 71

Discussion, 73

Chapter 5 Noradrenergic control of arginine vasopressin release from ewe hypothalamus *in vitro*: sensitivity to oestradiol, 76

Abstract, 77

Introduction, 77

Materials and methods, 79

Results, 80

Results, 121
Discussion, 123
Conclusions, 128

Chapter 10 Neuronal responses in the brainstem and hypothalamic nuclei following insulin treatment: delineating the mechanisms involved in the disruption of the GnRH surge in the ewe, 131
Abstract, 132
Introduction, 133
Materials and methods, 134
Results, 137
Discussion, 139
Conclusions, 148

Chapter 11 General discussion, conclusions and indications for future work, 152
General discussion, 153
Conclusions, 163
Indications for future work, 164

Bibliography, 166
Copies of publications

Abstract

Neuroendocrinology of stress and reproduction interaction

The aim of the present study was to examine central pathways and neurotransmitters through which stressors disrupt GnRH release, particularly via AVP under the influence of oestradiol (E_2). First, in an *in vitro* perifusion system for hypothalamic slices the effect of E_2 on GABA or noradrenergic control of AVP or GnRH release directly from ewe hypothalamic slices was investigated. Second, a confocal analysis of ewe hypothalamic sections was carried out to establish the presence of close contacts between various neurotransmitters systems involved in stress and reproduction interactions. Third, neuronal responses to *in vivo* insulin treatment using Fos-mapping of brainstem and hypothalamic nuclei delineated the activation of central mechanisms involved in stress-induced disruption of GnRH release.

The validity of the *in vitro* perifusion system was established by: 1) a robust release of AVP and GnRH in response to a depolarizing dose of KCl (100 mM) at the end of collection period, 2) histological evaluation of the slices which revealed intact neuronal cell bodies near the surface of slice.

Exposure to high E_2 (24 pg/ml) increased the release of AVP and GnRH from hypothalamic slices *in vitro* suggesting direct action of E_2 within the hypothalamus. A marked release of AVP in response to $GABA_{A\ or\ B}$ antagonists (10 mM) revealed an inhibitory GABA control over AVP release that was potentiated through $GABA_B$ receptors in the presence of E_2. However, stimulation of AVP release by both α_1-adrenoreceptor agonist and antagonist (10 mM) indicated the existence of dual stimulatory and inhibitory noradrenergic control over AVP neurones, with lower stimulatory response in the presence of E_2.

The release of GnRH in response to GABA receptor antagonists (10 mM) revealed predominant $GABA_A$ receptor-mediated inhibitory GABA control that was attenuated in the presence of E_2. However, GnRH release induced by an α_1-adrenoreceptor agonist (10 mM) was potentiated by E_2.

With confocal microscopy, close contacts were observed between noradrenergic terminals and CRH or AVP cell bodies in the PVN but not with β-endorphin cell bodies in the ARC. However, GABA terminals closely contact CRH and β-endorphin, but not AVP, cell bodies. No relation was observed between CRH, AVP and β-endorphin terminals and GnRH cell bodies in the mPOA, however, CRH and GnRH terminals were close in the ME. Oestradiol receptor (ERα) colocalisation was not observed in CRH and AVP neurones in the PVN but noradrenergic neurones in the brainstem and β-endorphin neurones in the ARC did colocalise ERα.

Following insulin treatment of ewes *in vivo,* there was robust Fos-immunoreactivity in the noradrenergic neurones in the brainstem and CRH and magnocellular and parvocellular AVP neurones in the PVN; however Fos decreased in the β-endorphin neurones in the ARC. The presence of Fos-positive cells increased in the ARC and the VMN after insulin. The colocalisation ERα and Fos-positive cells decreased in the ARC contrary to the increase in the VMN.

In conclusion, E_2-induced stimulation of AVP from the hypothalamus may be involved in E_2-induced augmentation of HPA activity, however, GABA and noradrenergic interactions with AVP neurones in the presence of E_2 do not favor this hypothesis. Oestradiol alone as well as in association with GABA and noradrenergic systems support the potentiation of GnRH release from hypothalamus *in vitro*. Complex interactions between various neurones in the brainstem and hypothalamus are involved in stress-induced suppression of the GnRH release.

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List of publications/presentations

Abstracts

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Abbreviations

A°	angstrom
A11, A12, A13, A14, A15	dopamine cell groups
ac	anterior commissure
ACTH	adrenocorticotrophic hormone
AHA	anterior hypothalamic area
AP	area postrema
ARC	arcuate nucleus
AVP	arginine vasopressin
dBNST	dorsal bed nucleus of stria terminalis
lBNST	lateral bed nucleus of stria terminalis
vBNST	ventral bed nucleus of stria terminalis
Bq	becquerel
CAG	chrom-alum-gel
cc	central canal
CO_2	carbon dioxide
cpm	counts per minute
cm	centimetre(s)
CMF	calcium magnesium free
CRH	corticotrophin releasing hormone
oCRH	ovine corticotrophin releasing hormone
Cy3	indocarbocyanine
dBB	diagonal band of Broca
DβH	dopamine β hydroxylase
DNH	dorsal nucleus of hypothalamus
dpm	disintegrations per minute
DTg	dorsal tegmental nucleus
E_2	oestradiol
EC	endcapped
EDTA	ethylenediaminetetraaceticacid

e.g.	for example
ER(α or β)	oestradiol receptor (alpha or beta)
FITC	fluorescein
Fx	fornix
g	gravitational force
GABA	γ-amino butyric acid
GAD	glutamine acid decarboxylase
GLM	general linear model
gm	gram(s)
GnRH	gonadotrophin releasing hormone
h	hour(s)
HBSS	Hanks's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N′-2 ethanesulfonic acid
HPA	hypothalamo-pituitary-adrenal
HPG	hypothalamo-pituitary-gonadal
HPLC	high pressure liquid chromatography
ID	internal diameter
IU	international unit
i.v.	intravenous
KCl	potassium chloride
Kg	kilograms
LC	locus coeruleus
LH	luteinising hormone
oLH	ovine luteinising hormone
LHA	lateral hypothalamus
LS	lateral septum
LSCM	laser scanning confocal microscopy
LV	lateral ventricle
M	molar
MB	mammillary bodies
MBH	mediobasal hypothalamus

ME	median eminence
MEM	minimum essential media
mg	milligrams
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MS	medial septum
Mt	mamillothalamic tract
MUA	multiunit activity
MW	molecular weight
NaI	sodium iodide
NEC	non-endcapped
ng	nannogram(s)
NPY	neuropeptide-Y
NTS	nucleus of solitary tract
O_2	oxygen
OC	optic chiasma
OR (μ, δ or κ)	opioid receptor (mu, delta or kappa)
OT	optic tract
OVLT	organum vasculosum of lamina terminalis
OVX	ovariectomized
PB	phosphate buffer
PBS	phosphate buffer saline
PeVA	periventricular area
PeVN	periventricular nucleus
pg	pictogram(s)
PHA	posterior hypothalamic area
pKa	dissociation constants
POA	preoptic area

lPOA	lateral preoptic area
mPOA	medial preoptic area
rPOA	rostral preoptic area
rmPOA	rostromedial preoptic area
vPOA	ventral preoptic area
vmPOA	ventromedial preoptic area
POMC	pro-opiomelanocortin
PR	progesterone receptor
PVN	paraventricular nucleus of hypothalamus
PVT	paraventricular nucleus of thalamus
Rch	retrochiasmatic area
RIA	radioimmunoassay
SCN	suprachiasmatic nucleus
SEM	standard error
SFO	subfornical organ
SON	supraoptic nucleus
SPE	solid phase extraction
TH	tyrosine hydroxylase
VLM	ventrolateral medulla
VMN	ventromedial nucleus
3V	third ventricle
4V	fourth ventricle
µg	microgram(s)
µm	micrometre(s)
~	around
<	less than
>	more than
%	percent
°C	degree celsius

Chapter 1

Introduction and literature review

Introduction

It is well documented that tightly regulated communications between the ovary and brain ensure that the neural signal for ovulation occurs when ovarian follicles are mature. A critical component of this communication system is the oestradiol (E_2) dependent regulation of GnRH hypersecretion that stimulates a preovulatory surge in LH release.

However, activation of hypothalamic-pituitary-adrenal (HPA) axis after exposure to hostile conditions (stressors) results in inhibition of reproductive physiology and sexual behavior especially the disruption of GnRH release. In the rat and ewe, it is proposed that during the stress-induced activation of HPA axis ovarian steroids, especially E_2, and GnRH signalling are disrupted, centrally.

Understanding the central mechanisms through which E_2 regulates, first, generation of GnRH surge, and second, potentiation of stress responses to inhibit GnRH has been the goal of numerous studies. As a result, researchers have found numerous brain regions and neurotransmitters that are regulated by E_2 and are important for GnRH regulation in response to changes in the environment.

This review of the literature will focus on the understanding of central pathways and neurotransmitters involved in mediating the ovarian steroid influence on neuroendocrine regulation of GnRH neurones.

Distribution of GnRH neurones

GnRH neurones (~2500 in the whole ovine brain) are not located in specific brain areas but are dispersed as loose networks. The axons of GnRH cell bodies project to two neurohaemal organs: the median eminence (ME) and the organum vasculosum of the lamina terminalis (OVLT) (Lehman *et al.*, 1986; Caldani *et al.*, 1988; Jansen *et al.*, 1997). The majority of GnRH cell bodies are found in the anterior portion of the hypothalamus with highest concentrations (~50-54%) in the medial preoptic area (mPOA) at the level of the OVLT. Remaining cell bodies form a loose continuum from the diagonal band of broca/medial septal region (dBB/MS, 14%) via the anterior/lateral hypothalamic area

(AHA/LHA, 16%) to the mediobasal hypothalamus [MBH, 15%; incorporating the ventromedial nucleus (VMN) and the arcuate nucleus (ARC)]. Outside the hypothalamic area, very few (5%) GnRH cell bodies and fibres are located in the olfactory bulbs, nervous terminalis, medial amygdala, mammillary bodies, mesencephalic structures or pars tuberalis (Caldani *et al.*, 1988; Skinner *et al.*, 1992). In rat and vole, these extra hypothalamic GnRH neurones have been associated with 'nonendocrine' functions such as control of sexual behaviour/response and olfactory cues (Pfaff, 1973; Dluzen *et al.*, 1981).

Axonal projections involved in regulating anterior pituitary function have origins within the preoptic hypothalamic area and reach the rostral end of the ME by two pathways: a major ventrolateral trajection above the optic tract in the AHA and LHA, and a less prominent medial periventricular route alongside the third ventricle (Lehman *et al.*, 1986). The OVLT, present on the dorsal surface of optic chiasma is another area involved in neurohaemal secretion of GnRH, though the concentration of immunoreactive terminals is much less than in the ME. In ewe, GnRH cell bodies located in a more anterior part of the preoptic area innervate the nonvascularised dorsal and vascularised ventral part of the OVLT (Caldani *et al.*, 1988). The ventral part of the OVLT contains a plexus of fenestrated capillary vessels. As there is no direct portal connection between the OVLT and anterior pituitary, the function of GnRH released into the general circulation remains unclear (McKinley *et al.*, 1983; Smith & Jennes, 2001).

GnRH neurones related to tonic/surge release

The vast network of GnRH neurones raises the question whether the final output to hypophyseal portal system is located in specific regions. Retrograde tract tracing from the ewe ME labelled 18-26% of GnRH cell bodies (neuroendocrine GnRH neurones) in each of hypothalamic areas (mPOA, dBB, MS, AHA, LHA and MBH) (Jansen *et al.*, 1997). This suggests that GnRH release into the portal circulation is due to the secretary activity of neuroendocrine GnRH neurones located in multiple areas. In fact, the absolute number of neuroendocrine GnRH neurones projecting from the mPOA to the ME is greater than from other areas, thus it is possible that this area may contribute in greater

proportion to final GnRH release. However, in the monkey and the rat, the low frequency of GnRH-GnRH synaptic interactions that occur throughout the hypothalamic areas (Chen *et al.*, 1990; Witkin *et al.*, 1995), throws into doubt a GnRH cell-to-cell co-ordination of widely scattered intrinsically pulsatile GnRH neurones. Therefore, GnRH output may be the result of synchronised activity of a neuronal network including GnRH and non-GnRH neurones along with non-neuronal cells such as glial and endothelial cells (Terasawa, 2001; Prevot, 2002). This scattered network of ovine GnRH neuroendocrine neurones does not rule out the existence of functional subsets of these neurones related to tonic release, preovulatory surge or both. Various methods have been used to identify specific areas with GnRH neurones responsible for these two different patterns of GnRH release; deafferentation studies, multiunit recordings and monitoring Fos.

Deafferentation studies

Bilateral knife cut studies in the hypothalamus predict that neurones located in the MBH are sufficient for tonic release of luteinising hormone (LH), whereas the LH surge is the result of output of GnRH neurones placed in the POA (Jackson *et al.*, 1978; Thiery *et al.*, 1978; Pau *et al.*, 1982). Knife cuts at the level of suprachiasmatic nuclei (SCN) attenuate the LH surge but do not prevent tonic LH release (Jackson *et al.*, 1978; Radford, 1979; Pau *et al.*, 1982; Pau & Jackson, 1985). Cuts between the SCN and the ARC block the LH surge and reduce LH amplitude without any effect on LH frequency (Jackson *et al.*, 1978; Thiery *et al.*, 1978; Thiery & Martin, 1991; Whisnant & Goodman, 1994). More caudally, deafferentation at the anterior edge of the ARC completely eliminates tonic and surge LH release (Jackson *et al.*, 1978; Radford, 1979).

Following deafferentation, severe or partial attenuation of tonic or surge LH release could be due to the relative proportions of the GnRH axons disrupted by a given knife cut in the ventrolateral or periventricular route projecting to the ME (Lehman *et al.*, 1986; Caldani *et al.*, 1988). This challenges the above prediction (Jackson *et al.*, 1978; Thiery *et al.*, 1978; Pau *et al.*, 1982) and suggests that control of tonic gonadotrophin release may not be limited to the MBH, because knife cuts at the SCN level might have spared many laterally placed GnRH axons in the ventrolateral route. It is further

postulated that the LH surge depends upon the coordinated activity of rostrally as well as caudally placed GnRH neurones (Lehman *et al.*, 1986).

Multiunit recordings

The recording of periodical bursts of multiunit electrical activity (MUA) from the hypothalamic pulse generator form the basis for the concept that the neural system fires periodically with high frequency burst action potentials that culminate in pulsatile GnRH neurosecretion (Knobil, 1981). MUA volleys are recorded by placing the tip of electrodes in the MBH, precisely the ARC-ME region, and suggest that the recording of volleys from the GnRH axonal route or from terminals is a product of coordinated firing of GnRH neurones. Alternatively, the absence of electrical discharge from the POA indicates that MUA volleys do not arise from the GnRH neurones themselves but from a neuronal oscillator located in the MBH, which activates GnRH neurones (Nishihara *et al.*, 1999). The original MUA studies in sheep could not derive any conclusive results (Rasmussen & Malven, 1981; Thiery & Pelletier, 1981). Later on, MUA volleys from the MBH were found to be coincident with LH pulses in monkeys, rats or goats (Wilson *et al.*, 1984; Kimura *et al.*, 1991; Ito *et al.*, 1993). Using simultaneous sampling, the time-lag between a GnRH pulse followed by an LH pulse was almost the same as that between a MUA volley and the succeeding LH pulse indicating that MUA volleys directly determine GnRH pulse frequency (Nishihara *et al.*, 1999).

During the oestrous cycle, MUA volley frequency from the MBH increases abruptly after the decline of progesterone concentrations and remains high during the follicular phase. However, before the preovulatory LH surge in intact goats, or an E_2-induced LH surge in ovariectomized (OVX) goats, MUA frequency decreases but without a change in baseline values providing the clue that the MBH neuronal circuitry (pulse generator) is primarily involved in the regulation of tonic LH release and the E_2-responsive neuronal circuitry (surge generator) which mediates positive feedback of E_2 is located in hypothalamic area(s) other than the MBH (Tanaka *et al.*, 1992; Tanaka *et al.*, 1995). Nevertheless, electrical activity and actual anatomical position of the surge generator is still unknown.

Fos studies

Fos is the protein product of an immediate early gene that is expressed transiently (to be maximal by approximately 2 h), typically following activation via a neurotransmitter or the neurohormone and declines thereafter (Hoffman *et al.*, 1993). Fos can be detected immunocytochemically in the cell nucleus of individual neurones after E_2 stimulation and is presumed to be a useful marker of GnRH neuronal activation in the ewe and other species although the precise association of Fos with GnRH biosynthesis release is not clear (Hoffman *et al.*, 1990; Lambert *et al.*, 1992; Moenter *et al.*, 1993). After E_2 treatment in OVX ewes, Fos is noticed within 1-2 h but for a transient duration (no Fos positive cells at 4 h) in the VMN and ARC in the hypothalamus and the caudal ventrolateral medulla (VLM) in the brain stem (Clarke *et al.*, 2001; Rawson *et al.*, 2001).

In the ewe, rat and mouse, little Fos expression is noticed in GnRH neurones at times other than the LH surge (Hoffman *et al.*, 1990; Wu *et al.*, 1992; Moenter *et al.*, 1993). In the luteal phase of ewe, during endogenous onset of LH pulses and during naloxone-induced stimulation of LH pulse frequency, expression of Fos in GnRH neurones (30-50%) is limited to only the MBH. This raises the possibility that a subset of GnRH neurones located in the ewe MBH may be sufficient to maintain pulse generation (Boukhliq *et al.*, 1999). However, at the time of the LH surge, a contribution of widespread GnRH neurones is suggested as Fos expression is observed in 40-70% of GnRH neurones located in all the hypothalamic areas, viz. dBB, POA, AHA or MBH (Moenter *et al.*, 1993; Richter *et al.*, 2001a).

Steroid receptor distribution and identity of steroid receptor-positive neurones

In the ewe, the role of gonadal steroid hormones viz., E_2 and progesterone, at the level of the pituitary gland in regulation of the preovulatory LH surge is well recognised (Goodman, 1994). In addition to the pituitary, the brain is another important site of gonadal steroid action involved in GnRH secretion (Tilbrook & Clarke, 1995; Clarke, 1995b). However, the exact location of steroid receptors, their action on GnRH neurones and the interaction between steroids within the brain to influence GnRH release into the portal circulation remains poorly understood.

A few years ago, in ewe and other species, immunohistochemistry and *in situ* hybridisation consistently failed to reveal steroid receptors in GnRH neurones. Furthermore, tritiated E_2 does not accumulate in the nuclei of GnRH neurones. However, double-labelling studies show that steroid receptor-positive cells surround GnRH neurones. These findings led to the conclusion that the action of steroid hormones on GnRH neurones is probably indirect through excitatory/inhibitory steroid-receptive neurones (Shivers *et al.*, 1983; Fox *et al.*, 1990; Leranth *et al.*, 1992; Herbison *et al.*, 1993b; Lehman & Karsch, 1993; King *et al.*, 1995; Herbison *et al.*, 1996; Laflamme *et al.*, 1998). In contrast, reports in recent years in the rat and guinea pig suggest that GnRH neurones may actually contain E_2 or progesterone receptors (Butler *et al.*, 1999; Skynner *et al.*, 1999; Hrabovszky *et al.*, 2000; Herbison & Pape, 2001; Hrabovszky *et al.*, 2001; Kallo *et al.*, 2001), but steroid receptors are still not detectable in ewe GnRH neurones (Skinner *et al.*, 2001).

Until the confirmation and establishment of these new findings, it is presumed that there is little or no direct regulation of GnRH neurones by steroids. Other evidence supports this; firstly blockade of the preovulatory LH surge by administration of neurotransmitter antagonists indicates that synaptic inputs of steroid-receptive neurotransmitters to GnRH neurones are involved in the regulation of GnRH secretion. Secondly, GT-1, a GnRH-secreting hypothalamus cell line, does not express E_2 receptor mRNA (Mellon *et al.*, 1990; Kalra, 1993; Terasawa, 2001).

Oestradiol receptor distribution

The original oestradiol receptor (ER) has now been renamed as ERα (Kuiper & Gustafsson, 1997) and the other newly identified E_2-specific receptor has been termed as ERβ (Kuiper *et al.*, 1996).

ERα

In sheep, using *in situ* hybridisation, ERα mRNA-positive cells occur in highest density (110-150 cells/mm^2) in the POA, the supraoptic and paraventricular nuclei (SON and PVN), the VMN and the ARC. A moderate density (80 cells/mm^2) of ERα mRNA-containing cells is located in the bed nucleus of the stria terminalis (BNST) and a low

density (30-50 cells/mm^2) is in the lateral septum (LS), subfornical organ (SFO), retrochiasmatic (Rch) area and the AHA (Scott *et al.*, 2000a). Using immunohistochemistry, a similar distribution of ERα-positive cells occurs in the ovine hypothalamus. However, in the SON and PVN, the cells are scattered, and no ERα-positive cells were found in the Rch (Herbison *et al.*, 1993b; Lehman *et al.*, 1993; Blache *et al.*, 1994). This discrepancy is not currently understood. ERα-positive cells are also in the amygdala, hippocampus and periaqueductal grey area along with the pars tuberalis and pars distalis (Skinner *et al.*, 1992; Lehman *et al.*, 1993). In the brainstem, ERα-positive cells are in the nucleus of solitary tract (NTS) and the ventrolateral medulla (VLM) (Scott *et al.*, 1998; Simonian *et al.*, 1998).

ERβ

The ovine SON, PVN, SFO and the Rch area has a high density (120-220 cells/mm^2) of ERβ mRNA-positive cells, whereas moderate concentrations (40-50 cells/mm^2) are present in the AHA, BNST, POA, VMN and the zona incerta. The LS and ARC do not contain ERβ mRNA-positive cells (Scott *et al.*, 2000a). Considerable overlapping of ERα and ERβ mRNA-positive cells detected in various brain nuclei indicate the interaction of receptor subtypes in the regulation of E_2 action. However, the little or no ERβ mRNA in the POA, VMN and ARC indicates that ERβ may not be involved in feedback actions of E_2 in these areas. Whereas the large population of ERβ mRNA-positive cells noted within the A15 dopaminergic cell group, raises the possibility of E_2 negative feedback through ERβ within this nucleus during anoestrus in ewe. Implantation of E_2 into the A15 nucleus attenuates LH release in OVX ewes during inhibitory photoperiod (Gallegos-Sanchez *et al.*, 1997; Scott *et al.*, 2000a). Similar ERβ mRNA distribution also occurs in the ram hypothalamus (Hileman *et al.*, 1999). However, the role of ERβ in the regulation of GnRH secretion is still not clear although the binding affinity of E_2 is the same for the both the receptor subtypes (Kuiper *et al.*, 1997). Male mice that lack ERβ are fertile and female mice provide litters, though with reduced frequency (Krege *et al.*, 1998).

Identity of ERα-positive neurones

Dopamine: The dopamine cell groups; A11, A13 (outside the PVN) and A15 (in the lateral Rch area) do not show any ERα in double-labelling studies in ewe (Lehman & Karsch, 1993; Skinner & Herbison, 1997). In the A14 group (in the POA alongside the third ventricle just dorsal to optic chiasm), 25% dopamine synthesising neurones possess ERα (Skinner & Herbison, 1997). Recently, however, a high density (150 cells/mm^2) of ERβ mRNA-positive cells was found in the A15 group (Scott *et al.*, 2000a). In the ARC, less than 10% ERα-positive neurones contain dopamine (Batailler *et al.*, 1992). From other studies, about 3 to 15% of dopamine neurones of the A12 group containing ERα are scattered throughout the ARC (Lehman & Karsch, 1993; Skinner & Herbison, 1997). Following E_2 treatment, between 9 to 30% dopamine neurones become Fos-positive in the ARC suggesting that they are E_2 responsive (Clarke *et al.*, 2001).

Noradrenaline: A large population of noradrenergic neurones positive for dopamine β hydroxylase (DβH; a marker for noradrenergic neurones) contain ERα, increasing in a rostrocaudal gradient in the VLM (from 4 to 19%) and NTS (from 8 to 21%) cell groups of the ewe brainstem (Simonian *et al.*, 1998). In the caudal VLM, approximately 73% ERα-positive cells are DβH-positive in comparison to 8% in cranial region. Whereas in NTS, about 9% ERα cells stain for DβH in the most caudal region then 2% in cranial part (Scott *et al.*, 1999).

γ-amino butyric acid (GABA): In ewe, about 44% neurones synthesising GABA colocalise with ERα in the POA and these account for 30% of all ERα-positive cells in this area (Herbison *et al.*, 1993b).

β-endorphin and Neuropeptide-Y (NPY): In the ARC, approximately 15-20% β-endorphin-positive neurones colocalise with ERα and about 3-10% NPY neurones possess ERα (Skinner & Herbison, 1997). Fos expression occurs in E_2-receptive β-

endorphin (<15%) and NPY (upto 30%) neurones of the ARC in E_2-treated OVX ewes (Clarke *et al.*, 2001).

Somatostatin: A large network of somatostatin-positive neurones is present in the ovine VMN and about 35% of these are ERα-positive (Willoughby *et al.*, 1995). In this area, approximately 70% ERα-positive cells synthesise somatostatin (Herbison & Fenelon, 1995). However, Fos response to acute E_2 challenge is not observed in these neurones (Clarke *et al.*, 2001).

Progesterone receptor distribution

The distribution of progesterone receptor (PR)-positive cells and PR mRNA-containing cells has recently been described in the hypothalamus of the OVX (Scott *et al.*, 2000b) and progesterone-treated OVX ewe (Skinner *et al.*, 2001). In the hypothalamus, PR-positive cells are detected with the highest density (60-90 cells/mm^2) in the mPOA, VMN and ARC. The periventricular nucleus (PeVN) has a moderate density (30 cells/mm^2) of PR-positive cells along with scattered cells in the SON and dBB (Scott *et al.*, 2000b; Skinner *et al.*, 2001). In other study, similar distribution of PR-positive cells occurs with the exception of the SON and the caudal ARC (below the mammillary recess) where PR-positive cells are not found (Skinner *et al.*, 2001). This could be due to different steroid treatment protocols. In the PVN and in any part of the brainstem, PR-positive cells have not been located. Further, PR mRNA-positive cells are only found in the hypothalamic nuclei, which contain PR-positive cells; and progesterone binding has been reported in the POA and the MBH (Bittman & Blaustein, 1990; Scott *et al.*, 2000b; Skinner *et al.*, 2001)

Identity of PR-positive neurones

The identity of PR-positive neurones in the ovine hypothalamus is not currently clear. Studies are required in this area to fully understand the regulation of GnRH secretion by progesterone in ewe, especially as progesterone regulates the action of various neurotransmitters in the brain, including GABA in the POA and dopamine, β-endorphin and enkephalin in the MBH (Whisnant & Goodman, 1988; Whisnant *et al.*,

1991; Robinson & Kendrick, 1992b; Broad *et al.*, 1993; Fabre-Nys *et al.*, 1994). Contrary to ewe, PR-positive cells are well described in the hypothalamus of rat, monkey and guinea pig. In these latter species, PR-positive cells colocalise with GABA in the POA, with dopamine neurones in the PeVN and with GABA, dopamine, β-endorphin, enkephalin and NPY in the ARC (Fox *et al.*, 1990; Olster & Blaustein, 1990; Leranth *et al.*, 1991; Kohama *et al.*, 1992; Warembourg *et al.*, 1994; Thind & Goldsmith, 1997).

Neuronal projections to GnRH cell bodies and terminals

For the regulation of GnRH secretion, steroid-receptive cells must communicate (directly) with GnRH neurones through synapses particularly in the mPOA, an area with a high density of GnRH cell bodies or through direct inputs to GnRH nerve terminals at the level of the ME (Lehman *et al.*, 1988a; Kuljis & Advis, 1989). However, meager evidence is available regarding the connections between the neuroanatomical framework of steroid-receptive cells and GnRH neurones.

In the ewe, retrograde tract tracing coupled with ERα double-labelling was used to map the origin of neuronal afferents reaching GnRH cells in the POA from various brain nuclei. However, neuronal tracer studies combined with PR double-labelling are not available. The injection of retrograde fluorescent tracer (fluorogold) in the rPOA or mPOA, labelled neurones in the LS, dBB, BNST, vPOA, PeV area, VMN, ARC and ME (Tillet *et al.*, 1993; Goubillon *et al.*, 1999). About 40% of these projections, containing ERα are located in the LS, POA, rostrocaudal VMN and ARC (Goubillon *et al.*, 1999). Also, a subset of E_2 positive neurones from the caudal VLM in the brainstem projects to the mPOA that contains maximum number of GnRH cell bodies (Tillet *et al.*, 1993; Goubillon *et al.*, 1999; Scott *et al.*, 1999; Rawson *et al.*, 2001). Precise injection of retrograde tracer into the rostroventral part of the mPOA near to the OVLT labels cells in the brainstem; however, injections immediately around this site result in little or no labelling in the brainstem (Scott *et al.*, 1999). After injection of retrograde tracer in the VLM, a few labelled cells are also located in the VMN (Rawson *et al.*, 2001).

Recent anterograde tracing studies show that most projections (345 terminals/mm^2) from the ERα rich MBH (ARC/VMN) extend to the POA, BNST and

AHA, and a few (33 terminals/mm^2) to the PVN, but only 1.3% of afferents in the POA are in close contact with GnRH cell bodies (Pompolo *et al.*, 2001). Another, anterograde tracing study from the VMN shows a dense network of labelled fibres in the dBB, POA, AHA, dorsomedial nuclei, PeVN and PVN and a low density is detected in septal region, BNST, thalamus PVN, LHA and ARC (Goubillon *et al.*, 2002). But in contrast to former anterograde study, in another study a large number of GnRH neurones in the dBB (46%), POA (55%), AHA (57%) and MBH (62%) are found in close proximity to the VMN projections. The reason for this discrepancy is unclear. However, these findings suggest that neurones in the MBH (VMN) are capable of either directly regulating the GnRH neurones or via the existence of a local interneuronal network in these areas which receive signals from the MBH and convey with or without modulation onward to GnRH neurones. Although a dense population of GnRH neurones is present in the OVLT, there are no projections from the ARC/VMN to this area (Lehman *et al.*, 1986; Lehman & Karsch, 1993; Pompolo *et al.*, 2001; Goubillon *et al.*, 2002). Regarding input to the Rch area, retrograde tracing shows that ERα cells projecting to the Rch area originate from the AHA, rmPOA, VMN and ARC (Jansen *et al.*, 1999).

To examine the possibility of direct modulation of GnRH secretion by ERα cells at the level of the ME, retrograde tracer injections into the ME reveals a low proportion of ERα-positive neurones projecting to the ME from the OVLT and ARC and not from the remaining E_2-rich perikarya of the hypothalamus (Jansen *et al.*, 1996). In agreement, very few anterogradely labelled fibres from the VMN to the ME suggest a limited role for VMN neurones in modulation of GnRH nerve terminal activity (Goubillon *et al.*, 2002).

Locus of steroid action and interaction in the brain to regulate GnRH secretion

Negative and positive feedback effects of steroids

During the luteal phase, progesterone prevents the positive feedback effect of E_2 on GnRH secretion. After luteolysis, the progesterone negative feedback is removed and the follicular phase is characterised by an increase in E_2 secretion and GnRH pulse frequency (Moenter *et al.*, 1990; Clarke, 1995a). The increasing E_2 concentrations exert positive feedback actions in the hypothalamus resulting in increase of GnRH secretion,

which leads to generation of the preovulatory LH surge (Evans *et al.*, 1994; Karsch *et al.*, 1997). To control this dual regulation of GnRH secretion, E_2 and progesterone act in synergy during the oestrous cycle (Goodman *et al.*, 1981). The mechanism by which E_2 and progesterone control timing of events in the ewe oestrous cycle is associated with modulation of GnRH release by mechanism other than marked alteration in GnRH gene expression (Harris *et al.*, 1998; Robinson *et al.*, 2000). However, dynamic changes in preoptic GnRH transcriptional activity are involved in modulating parameters of steroid-induced LH surge in the rat (Jimenez-Linan & Rubin, 2001). The role of ERs and PRs seems to be crucial and the specific E_2-responsive elements are seen in the promoter of the PR gene in rat (Kraus *et al.*, 1993). The interactions of steroids may occur within the same cell as the PR-positive cells are observed in ERα positive cells though not all ER-positive cells express PR in the guinea pig (Blaustein & Turcotte, 1989; Warembourg *et al.*, 1989). In ewe, all PR-positive cells in the POA and ARC are ERα-positive, suggesting that under varying steroid milieu, a single cell could synthesise different receptors that may exert opposing effects on GnRH secretion (Dufourny & Skinner, 2001).

Progesterone has both stimulatory and inhibitory effects on the GnRH release. Continuous presence of progesterone during the follicular and preovulatory phases prevents the positive feedback of E_2 on GnRH/LH secretion (Kasa-Vubu *et al.*, 1992). The increased duration and amplitude of luteal phase progesterone exposure delays the responsiveness of LH surge generating system without any effect on surge amplitude or duration, after constant E_2 stimulus (Skinner *et al.*, 2000). Also, the delay in the E_2 surge is longer in ewes with prior exposure to two progesterone implants in comparison to ewes with one implant (Van Cleef *et al.*, 1998). On the other side, the magnitude of the E_2-induced GnRH surge is attenuated in absence of progesterone priming suggesting the physiological role of progesterone in full expression of preovulatory gonadotrophin surge (Caraty & Skinner, 1999).

The effects of progesterone regimes that either augment (progesterone pre-treatment) or inhibit (progesterone treatment at the time of surge-inducing E_2 increment) the E_2-induced GnRH surge have been delineated using steroid replacement in OVX ewes. Augmentation appears to be through Fos-activation in non-GnRH cells, without any

effect on the proportion of activated GnRH neurones; however, the inhibition of the GnRH surge prevents the activation of both GnRH neurones and non-GnRH cells. This suggests that the divergent effects of progesterone on GnRH release are mediated via two different systems (Richter *et al.*, 2001a). The stimulatory effect of progesterone may be through induction of ERα. In the OVX ewes, E_2 treatment has no effect on ERα cell populations in the hypothalamus, however, ERα are apparent after progesterone plus E_2 treatment (Blache *et al.*, 1994). Further, non-GnRH cellular activation in progesterone-pretreated ewes is not essential for the expression of LH surge and may show activation of neural substrates linked with oestrous behavior (Lehman *et al.*, 1994; Richter *et al.*, 2001a). The suppressive action of progesterone on tonic and surge GnRH release may be mediated via nuclear PRs (Skinner *et al.*, 1998a) and/or through down regulation of ERα expression following long exposure to progesterone (Scaramuzzi *et al.*, 1971; Fabre-Nys & Martin, 1991; Skinner *et al.*, 1998a; Caraty & Skinner, 1999; Skinner *et al.*, 1999a). In addition, progesterone directly inhibits expression of E_2-induced genes *in vitro* (Meyer *et al.*, 1989). Progesterone can also suppress neuronal systems activated by E_2 during the LH surge (discussed under GnRH surge-induction process).

Further, E_2 enhances the suppressive action of progesterone on tonic LH secretion (Brann & Mahesh, 1991; Burke *et al.*, 1996; Girmus *et al.*, 1996). In OVX goats, progesterone in combination with E_2 suppresses MUA volley frequency but progesterone alone is ineffective (Tanaka *et al.*, 1994). In fact, no change in PR concentration occurs during the luteal or follicular phase of the ewe oestrous cycle, but PR mRNA increases at oestrus prior to the LH surge suggesting that the abrupt rise in E_2 at the time of the surge is responsible for increased PR mRNA expression. This increase in PR mRNA may be required for rise in PR-protein early in the luteal phase (Scott *et al.*, 2000b). Thus, expression of PRs is up regulated by E_2 and not by progesterone. Also, E_2 treatment increases the number of PR mRNA, PR-positive cells or amounts of PR binding, throughout the hypothalamus in number of species including ewe (Bittman & Blaustein, 1990; Leranth *et al.*, 1992; Bethea *et al.*, 1996; Shughrue *et al.*, 1997b; Scott *et al.*, 2000b). Moreover, PR-immunoreactivity is less intense in OVX ewes receiving only progesterone than those administered with both ovarian hormones (Dufourny & Skinner, 2001). Therefore, an E_2-stimulated increase in PRs may be involved in the synergistic

steroid interaction of negative feedback that controls GnRH pulse frequency during the luteal phase.

Site of steroid action

Precisely where and how these steroids interact to mediate effects in the central nervous system remains unsolved. Where does progesterone act to inhibit GnRH secretion and ultimately LH pulse frequency? The identity of the neurones and the exact pathway by which E_2 triggers preovulatory GnRH and LH surge is also undefined. As already discussed, ovarian steroid action on GnRH neurones is believed to be indirect because GnRH neurones do not possess E_2 or progesterone receptors (Fox *et al.*, 1990; Leranth *et al.*, 1992; Lehman *et al.*, 1993; Lehman & Karsch, 1993; King *et al.*, 1995; Skinner *et al.*, 2001) although this is now being questioned (Butler *et al.*, 1999; Skynner *et al.*, 1999; Hrabovszky *et al.*, 2000; Herbison & Pape, 2001; Hrabovszky *et al.*, 2001; Kallo *et al.*, 2001).

The ERs and PRs are present in overlapping hypothalamic areas such as the POA and the MBH but evidence is gathering in support of the MBH as the potential target site for steroid interaction(s) to ultimately control GnRH secretion profiles. Implantation of E_2 in the region of the VMN within the MBH of OVX ewe evokes a preovulatory LH surge (Blache *et al.*, 1991; Caraty *et al.*, 1998a) whereas progesterone implants or microinfusion in the VMN of OVX ewes blocks the E_2-induced LH release (Blache *et al.*, 1996; Skinner *et al.*, 1999a). Surprisingly, despite the presence of the highest density of GnRH neurones in the POA (Lehman *et al.*, 1986), implants of progesterone or E_2 in that area are not able to alter GnRH/LH release (Blache *et al.*, 1991; Blache *et al.*, 1996; Caraty *et al.*, 1998a; Skinner *et al.*, 1999b). In progesterone-treated OVX ewes, ERα increases in the VMN but not in other regions of the hypothalamus (Blache *et al.*, 1994). In comparison to luteal and follicular phase ewes, expression of PR mRNA concentration/cell is more in oestrous ewes in the VMN and ARC along with higher numbers of PR positive cell/mm^2 in the VMN but not the ARC. No cyclic variation is observed in PR expression in the POA (Scott *et al.*, 2001). More specifically, in OVX ewes, E_2 treatment significantly increases PR positive cells (by 60-90%) as well as PR mRNA (by 84-148%) in the VMN and ARC. However, a smaller increase (17-33%) in

PR positive cells/mm^2 without any effect on the PR mRNA concentration/cell is noticeable in the POA and the PeVN (Scott *et al.*, 2000b).

In summary, these studies in ewe indicate: first, VMN and ARC as primary sites for steroidal interactions that control the tonic/surge GnRH release. Second, progesterone and E_2 do not act directly in the POA but interact with cells in the MBH region, which in turn regulate GnRH secretion. Third, the role of the POA is unclear as GnRH neurones and non-GnRH cells in the POA/AH expresses Fos after progesterone administration (Richter *et al.*, 2001a). Progesterone implants in the POA in OVX ewes increased the release of GABA and decreases the release of noradrenaline in the POA, whilst peripheral progesterone administration had no effect (Robinson & Kendrick, 1992a) suggesting a direct effect. It remains to be explained exactly how this interaction between steroid-receptive cells in the MBH which project/relay to GnRH perikarya in the POA is regulated. After administering E_2 to OVX ewes, Fos immunohistochemistry of the MBH reveals that the majority of cells producing Fos are of unknown phenotype (NPY-, ACTH-, TH-positive neurones account for only 30% of total Fos positive cells). Whether this Fos is related to negative or positive E_2 feedback remains unknown (Clarke *et al.*, 2001).

Evidence for genomic or non-genomic pathway of steroid action

The mechanism of steroid action on GnRH neurones or non-GnRH cells involved in GnRH release may occur in a genomic or non-genomic way. Genomic effects are delayed (several hours to days) and involve action of the ovarian hormones via specific nuclear receptors leading to gene transcription (Chabbert-Buffet *et al.*, 2000; Falkenstein *et al.*, 2000). The chances of direct genomic action of ovarian steroids on GnRH cell population are meagre because of the absence of steroid-receptors in GnRH neurones. The genomic effects of progesterone through the classic PR-dependent pathway are revealed using the antiprogestin RU486 (Mifepristone), which reverses progesterone-induced inhibition of GnRH/LH pulse frequency (Skinner *et al.*, 1998b; Skinner *et al.*, 1999a). However, a potent suppressive action of progesterone treatment on GnRH pulse frequency occurs within 50 min, which indicates non-genomic action (Skinner *et al.*, 1998a). The well-supported non-genomic actions of steroids upon neuronal activity are

rapid (within minutes) via membrane effects through interaction of steroids with ion channels or via neurotransmitter receptors (Schumacher, 1990; McEwen, 1991; Chabbert-Buffet *et al.*, 2000; Falkenstein *et al.*, 2000). Recent electrophysiological findings suggest a rapid direct action of allopregnanolone (5α-pregnan-3α-ol-20-one), a progesterone neuroactive metabolite, upon native GnRH neurones in mice (Sim *et al.*, 2001). Following bolus administration of E_2 in rats, the firing rate of neurones in the POA changes within 5 min (Yagi, 1973). In OVX ewe, after E_2 implant, negative feedback on GnRH pulse amplitude is immediate (within 2 h) whereas the positive feedback on GnRH pulse frequency leading to surge generation is time-delayed (by 16-20 h) (Caraty *et al.*, 1989; Evans *et al.*, 1995a; Evans *et al.*, 1995b). The rapid suppressive effect of E_2 is probably non-genomic though the precise mechanism is not clear in ewe. The prolonged E_2 exposure required to generate an LH surge suggests a genomic action, which alters gene expression through the onset of a train of neural events, which involve the coordinated activity of GnRH neurones and various neurochemical inputs from non-GnRH cells. In addition, non-genomic action of E_2 has no role in surge onset, as elevated E_2 is not required at the time of surge (Evans *et al.*, 1997; Herbison, 1998).

Neurotransmitters

In the ewe hypothalamus, it is unlikely that effects of E_2 and progesterone are exerted directly on GnRH neurones at a classical nuclear receptor because GnRH neurones not exhibit this type of steroid-receptors. Therefore, anatomical substrates for modulatory influence of ovarian steroids on GnRH secretion are intermediary neurones containing steroid receptors. From immunohistochemistry it appears as though the major regulaton of the tonic and surge release of GnRH is coordinated via GABA, noradrenergic and opioidergic systems. These are the priniciple systems considered in this thesis, however, this is not meant to imply that other systems may be important.

γ-amino butyric acid

γ-amino butyric acid neurones, as well as a dense plexus of fibres containing GAD (Glutamic acid decarboxylase, a GABA biosynthetic enzyme) are located within the ewe POA and SCN (Herbison *et al.*, 1993b; Jansen *et al.*, 1994). In the rat, GABA neurones

are also present in the MBH and in the external layer of the ME. Additionally, in this area mRNAs code for GABA receptor subunits along with high affinity uptake sites for GABA (Tappaz *et al.*, 1980; Anderson & Mitchell, 1986b; Araki *et al.*, 1992; Horvath *et al.*, 1997; Ovesjo *et al.*, 2001). In the POA, GABA may directly control GnRH perikarya as GAD terminals synapse directly on GnRH cell bodies in the ewe and rat, $GABA_A$ receptor mRNA colocalises with GnRH neurones, and electrophysiological studies reveal functional $GABA_A$ receptors on all GnRH neurones in the rat (Herbison *et al.*, 1998; Jung *et al.*, 1998; Spergel *et al.*, 1999; Sim *et al.*, 2000).

In the POA and MBH, GABA acts through one or both of the two-receptor subtypes, $GABA_A$ and $GABA_B$. A large body of evidence propose that GABA acts through activation of $GABA_A$ receptors in the sheep POA and MBH to suppress gonadotrophin secretion, whereas, results of $GABA_B$ receptor activation are not so simple. There is no effect of $GABA_B$ receptor activation in the POA but LH pulse amplitude is stimulated by $GABA_B$ receptors in the MBH (Scott & Clarke, 1993a; Scott & Clarke, 1993b; Ferreira *et al.*, 1996; Ferreira *et al.*, 1998). This stimulatory effect is due to autoreceptor type activity of the $GABA_B$ receptor. Local stimulation of autoreceptors reduces secretion of endogenous GABA and thus releases GnRH neurones from inhibition (Hileman & Jackson, 1999).

Oestradiol influence on GABA neurones; direct or indirect

A negative correlation between LH and GABA release provides strong evidence for a predominant inhibitory influence of GABAergic neurones on GnRH neurones (Robinson, 1995). Oestradiol receptive GABA neurones could mediate E_2 negative feedback on GnRH neurones as GABA concentrations are increased in the POA by E_2 treatment of OVX ewes. The other principal change in the GABA picture is the marked decrease in GABA tone before the generation of an E_2-induced preovulatory LH surge in ewe (Robinson *et al.*, 1991). This hypothesis is further supported by a decrease in GAD mRNA expression in the rat POA before the onset of an endogenous or E_2-induced LH surge, and by the blockade of the surge after infusion of GABA in the POA or the third ventricle (Morello *et al.*, 1989; Herbison & Dyer, 1991; Herbison *et al.*, 1992; Unda *et al.*, 1995). In addition, the biphasic action of E_2 may be modulated by GABA neurones to

produce the characteristic profile of LH secretion, in the late follicular phase of the oestrous cycle. Oestradiol attenuates the autoinhibition of GABA POA neurones during the initial LH suppressed (negative feedback) phase and subsequently reduces GABA tone during the LH surge (positive feedback) (Wagner *et al.*, 2001). The start of a slow decline in GABA concentrations well in advance (by 10 h) of first rise in the LH surge indicates the permissive nature of GABA neurones (Robinson *et al.*, 1991).

A direct action of E_2 upon GABA neurones to enhance inhibitory GABA effects is suggested by E_2 stimulated increase in GABA turnover/reuptake, increases in $GABA_A$ receptor expression and colocalisation of ERs on GABA neurones (Herbison, 1997a).However, the 2 h time-delayed increase in basal GABA concentration within the POA following E_2 treatment, suggests the actions of E_2 on GABA neurones via interneuronal network (Herbison & Dyer, 1991). Noradrenergic systems are likely to mediate E_2 influence on GABA network (see under noradrenaline).

Progesterone influence on GABA neurones; direct or indirect

Progesterone is another primary hormonal signal that regulates the ewe GnRH-LH axis. Evidence indicates that opioids mediate the suppressive effects of progesterone on GnRH neurones, though noradrenaline and GABA are also involved (Whisnant *et al.*, 1992; Robinson & Kendrick, 1992a; Richter *et al.*, 2001). In ewes, there is sufficient evidence to implicate GABA neurones in some of the suppressive effects of progesterone on tonic GnRH release. First, progesterone treatment of OVX ewes in the breeding season elevates GABA concentrations in the POA. Second, progesterone suppresses the E_2-induced preovulatory surge of LH by maintaining elevated concentrations of the inhibitory GABA, and by depressing stimulatory noradrenaline elements in the POA (Robinson & Kendrick, 1992a).

In the guinea pig, PRs are identified on GABA neurones (Blaustein & Turcotte, 1989; King *et al.*, 1995). Functional $GABA_A$ receptors are present on GnRH neurones in the rat. Hence, a direct interaction of progesterone with GnRH and/or GABA neurones can be investigated using the neuroactive metabolite, allopregnanolone a potent allosteric modulator of the $GABA_A$ receptor (Baulieu, 1997). Activation of the $GABA_A$ receptor by allopregnanolone suppresses GnRH secretion and blocks ovulation by attenuating LH

release in the rat (Genazzani *et al.*, 1995; Calogero *et al.*, 1998). Also, progesterone modulates electrical activity of GnRH neurones through interaction of its neuroactive steroid with $GABA_A$ receptors (Sim *et al.*, 2001). Work in E_2-primed OVX ewe fails to detect any effect of intracerebroventricular infusion of allopregnanolone on GnRH/LH pulse frequency thus ruling out a direct role of $GABA_A$ receptor-allopregnanolone interaction though progesterone acts via its receptors (Skinner *et al.*, 1998a; Skinner *et al.*, 1999a). Therefore, other interneuronal systems viz. opioids may mediate the effects of progesterone on GABA neurones. In the rat, GABA neurones form synaptic contacts with POMC cell bodies in the ARC (Fox *et al.*, 1990; Horvath *et al.*, 1992; Blasquez *et al.*, 1994).

GABA verses seasonal anoestrus

Induction and/or alteration in function of GABA receptor subtypes is linked with a seasonal switch in the degree of E_2-feedback. The probable reason is the changes between active-inactive states of $GABA_A$ receptor, which are influenced by E_2 (Schumacher & McEwen, 1989). During the breeding season, $GABA_A$ but not $GABA_B$ receptor manipulations in the POA inhibit LH secretion, in both OVX and E_2-treated OVX ewes. This suggests that GABA neurones are not integrally involved in mediating the E_2 negative effect on GnRH secretion at this time. In contrast, during the non-breeding season, under the enhanced negative feedback effects of E_2 on LH, $GABA_A$ and $GABA_B$ receptor manipulations increase LH pulse amplitude in E_2-treated OVX ewes only (Scott & Clarke, 1993a; Scott & Clarke, 1993b). Similarly, application of $GABA_B$ agonist within the MBH increases LH pulse amplitude without any affect on LH pulse frequency in castrated rams during anoestrus (Ferreira *et al.*, 1996; Jackson *et al.*, 2000), implicating the GABA network in the mediation of negative feedback effect of E_2 during anoestrus. An other possible explanation for these observations is the relative inactivity of the GABA system during the breeding season as indicated by low GABA content in the POA compared with anoestrus, regardless of E_2 treatment (Viguie *et al.*, 1996). Conversely, during non-breeding season, when photoperiod-induced GABA activity is increased then GABA systems may become more sensitive to GABA receptor manipulations (Hileman & Jackson, 1999).

Noradrenaline

Stimulatory role of noradrenaline

Noradrenergic systems have a stimulatory role during surge induction process but a suppressive effect on tonic LH release (Parvizi, 2000). Associated with the preovulatory surge, an increase in noradrenaline release occurs in the POA and ARC-ME in the rat and ewe (Demling *et al.*, 1985; Domanski *et al.*, 1991; Mohankumar *et al.*, 1994; Clarke *et al.*, 1999). Also, administration of α-adrenoreceptor antagonists consistently blocks the E_2-induced LH surge in rats (Herbison, 1997b). Since brainstem noradrenergic neurones colocalise ERα, E_2 could directly activate noradrenergic neurones to transduce feedback actions (Simonian *et al.*, 1998; Scott *et al.*, 1999). Oestradiol increases Fos (within 1-2 h) and electrical excitability of brainstem noradrenergic neurones projecting to the POA (Kaba *et al.*, 1983; Clarke, 1995a; Rawson *et al.*, 2001). A positive correlation between E_2 concentrations and noradrenaline release in the POA and the ME suggests that noradrenaline is pivotal in mediating the stimulatory action of E_2 on the GnRH network (Herbison, 1998; Smith & Jennes, 2001). In the ME of monkeys, noradrenaline pulses drive GnRH pulses (Terasawa *et al.*, 1988), but no temporal correlation exists between noradrenaline and LH pulses in the ovine POA (Robinson *et al.*, 1991; Robinson & Kendrick, 1992a). Also, inability of α-adrenoreceptor blocker to alter GnRH or LH pulse frequency in E_2-treated OVX ewes puts question on positive correlation (Goodman *et al.*, 1996).

Suppressive role of noradrenaline

The suppressive role of noradrenaline in tonic gonadotrophin release is revealed by local administration of an α-adrenoreceptor antagonist in the POA to increase LH pulse amplitude in breeding season ewes (Goodman *et al.*, 1996). Moreover, noradrenergic input to the POA mediates the E_2-induced inhibition of GnRH pulse amplitude, as implants of α-adrenoreceptor antagonist into the POA increase pulse amplitude in E_2-treated OVX ewes but not in OVX ewes (Goodman *et al.*, 1996). In contrast, after peripheral administration of α-adrenoreceptor antagonist, LH pulse

amplitude decreases both in OVX and E_2-treated OVX ewes (Meyer & Goodman, 1986; Goodman *et al.*, 1996). Thus, during the breeding season, noradrenergic neurones are both stimulatory and inhibitory to GnRH neurones through influence at multiple areas in the brain.

Oestradiol influence on noradrenergic neurones; direct or indirect

Detailed investigations have started to reveal the origin of noradrenergic pathways to the POA through which E_2 controls GnRH secretion. Dense innervation of the POA by noradrenergic neurones is demonstrated by DβH-immunostaining (Lehman *et al.*, 1988b; Tillet *et al.*, 1989). Retrograde tracing studies show that the noradrenergic neuronal input to mPOA arises exclusively from cells in the caudal field of VLM (Rawson *et al.*, 2001). In the caudal VLM, the colocalisation of noradrenergic neurones with ERα and expression of Fos after E_2 treatment strongly supports the importance of noradrenergic neurones projecting from the caudal VLM nucleus to the POA for E_2-mediated regulation of GnRH (Simonian *et al.*, 1998; Scott *et al.*, 1999; Rawson *et al.*, 2001). However, no projections can be traced from the POA to the E_2-receptive noradrenergic neurones in the NTS (Tillet *et al.*, 1993; Rawson *et al.*, 2001).

Importantly, E_2 implants in the VMN induce the gonadotrophin surge (Blache *et al.*, 1991; Caraty *et al.*, 1998a). In the ARC-ME region, heavy noradrenergic innervation occurs (origin unknown) and a direct neuronal link (characteristics unknown) between the VMN and VLM cell bodies is revealed by retrograde tracer studies (Tillet *et al.*, 1989; Rawson *et al.*, 2001). Furthermore, a marked increase in noradrenaline release and dopamine metabolites from the ARC-ME and VMN respectively occurs at the time of E_2-induced LH surge (Kaynard *et al.*, 1990; Domanski *et al.*, 1991; Anderson *et al.*, 2001b). Therefore, considerable potential for indirect activation of brainstem noradrenergic neurones through afferents situated in the VMN and E_2-receptive dopamine neurones (pathway unknown) are probably involved. In contrast, others do not find changes in noradrenaline release, noradrenaline metabolite concentrations or DβH immunostaining in the MBH around the time of an E_2-induced LH surge in OVX ewes (Fabre-Nys *et al.*, 1994; Clarke *et al.*, 1999; Anderson *et al.*, 2001b). Lastly, whether E_2 acts directly on

brainstem noradrenergic neurones or through neurones located in the MBH remains to be resolved.

Pathway of noradrenergic-GnRH neuronal interaction

What is the exact mode of interaction between brainstem noradrenergic terminals and GnRH neurones in the mPOA? Electron microscopy reveals synaptic contacts between GnRH neurones and noradrenergic terminals in the rat (Leranth *et al.*, 1988a; Chen *et al.*, 1989b), but in ewe evidence for such close contacts in the mPOA is available in ewe at the confocal level (Pompolo *et al.*, 2003a). In the rat, α-adrenoreceptors are present in GnRH neurones as well as in GT-1 cell-lines (Findell *et al.*, 1993; Lee *et al.*, 1995; Hosny & Jennes, 1998). The possibility also exists for the indirect influence of noradrenergic terminals on GnRH neurones because the GnRH cell bodies are predominantly present in the mPOA, but reduction in DβH staining is observed in the dPOA as well as vPOA, which suggests the participation of interneuronal network in noradrenergic action (Lehman *et al.*, 1988b; Clarke *et al.*, 1999). In the rat, GnRH neurones receive inputs from GABA neurones, which have direct contact with noradrenergic fibres from the brainstem (Leranth *et al.*, 1988a). Microdialysis studies indicate direct regulation of GABA neurones by the brainstem noradrenergic neurones (Herbison *et al.*, 1990). All these findings suggest that noradrenaline may affect GnRH neurones in the POA via direct or indirect interactions or both may be involved. No ultrastructural evidence is available in ewe for influence of noradrenaline on GnRH terminals in the ME.

Is noradrenaline a trigger or permissive for gonadotrophin surge?

It is well known that during the follicular phase of the oestrous cycle and in E_2-treated OVX ewes, the LH surge is caused by a time-delayed biphasic response to E_2 (Caraty *et al.*, 1989; Clarke, 1995a; Walsh & Clarke, 1996; Anderson *et al.*, 2001a). The question now is whether the immediate Fos responses (within 1-2 h) in noradrenergic neurones in the brainstem are concerned with early negative (within 2 h) or subsequent positive feedback (by 16-20 h) of E_2 (Caraty *et al.*, 1989; Anderson *et al.*, 2001b; Rawson *et al.*, 2001). After E_2 injection, no change in DβH immunostaining in the mPOA is

noticeable for 6 h but a marked decrease follows before (at 16 h) the time of the surge (Clarke *et al.*, 1999). More specifically, in E_2-treated ewes, higher preoptic noradrenaline pulsatile release occurs in the 8 h period before the onset of the surge (Robinson *et al.*, 1991).

Another important concept is that noradrenaline release is not a trigger but is permissive for the GnRH surge (Robinson *et al.*, 1991; Clarke, 1995a; Clarke *et al.*, 1999). The validity of this permissive concept is emphasized by: first, the time difference (12 h) between maximum preoptic noradrenaline concentration and the onset of surge in ewe (Robinson *et al.*, 1991). Second, the absence of one-to-one correlations between preoptic noradrenaline and LH pulses in rat (Jarry *et al.*, 1990). Third, the temporal relation between noradrenaline, GABA and the LH surge: following E_2 treatment in OVX ewes, activation of noradrenaline neurones in the brainstem leads to an increase in pulsatile noradrenaline release within the POA, throughout the period leading to the onset of the surge. Initially, during E_2 negative feedback, noradrenaline stimulates GABA, which ultimately suppresses gonadotrophin release, but during positive feedback in the preovulatory period, noradrenaline release does not activate GABA neurones. The precise reason for uncoupling of the noradrenaline stimulatory influence upon GABA neurones prior to the LH surge is not clear. Nevertheless, this mechanism seems to release the GnRH perikarya from inhibition, thus permitting the LH surge to occur (Herbison *et al.*, 1990; Robinson *et al.*, 1991; Herbison, 1997a). Moreover, opioids are known to inhibit preoptic noradrenaline release in the rat. During the presurge period, the decrease in opioid tone may also contribute to increased noradrenaline outflow in the POA (Kalra & Kalra, 1984; Bicknell, 1985; Diez-Guerra *et al.*, 1987).

Opioids

Pro-opiomelanocortin (POMC), pro-enkephalin and pro-dynorphin are the classic precursors of opioids in the hypothalamus, which give rise to β-endorphin, met-/leu-enkephalin and dynorphins-A/B, respectively (Parvizi, 2000). The β-endorphin and POMC mRNA expressing neurones are exclusively located throughout the ovine ARC. A dense network of enkephalin-positive neurones is present in the VMN, SCN, and

hypothalamic PVN as well as in the ME, whereas pro-enkephalin mRNA expressing neurones are found widely distributed in the ewe hypothalamus, especially in the MS, dBB, POA, AHA, PVN, PeVN and VMN. A high density of dynorphin-A-positive neurones occurs in the SCN and SON. Endorphin-positive terminals from the ewe ARC project extensively to the mPOA and external zone of ME and are also found close to GnRH neurones in the periventricular area, whilst the projections of enkephalin-positive neurones have not been mapped in detail in the ewe (Marson *et al.*, 1987; Tillet *et al.*, 1989; Giraud *et al.*, 1991; Matthews *et al.*, 1992; Whisnant *et al.*, 1992; Broad *et al.*, 1993; Conover *et al.*, 1993; Lehman & Karsch, 1993; Walsh *et al.*, 1998; Henry *et al.*, 2000; Walsh *et al.*, 2001).

Information concerning the involvement of ewe enkephalin and dynorphin fibres in gonadotrophin secretion is not available. However, many studies have attempted to unmask the role of β-endorphin neurones in transmitting the input of ovarian steroids to GnRH neurones (Herbison, 1998). The various opioid effects on GnRH neurones are mediated through three well-known opioid receptor (OR) subtypes designated as mu (μ-), delta (δ-) or kappa (κ-) and are encoded from the genes; Mor-1, Dor-1 and Kor-1 (Parvizi, 2000). The ORs exist in two potential sites of opioid action in the ewe hypothalamus viz., POA and MBH. The density of ORs in the MBH is lower than that in the POA but the distribution of OR subtypes is similar (Shen *et al.*, 1995). Recently, ORμ mRNA expressing neurones were demonstrated throughout the ewe hypothalamus (MS, dBB, mPOA, AH and BNST; (Walsh *et al.*, 2001).

The extent to which opioids regulate GnRH secretion is demonstrated by monitoring gonadotrophin responses to a challenge with opioid agonists or antagonists. Agonists, such as morphine, suppress tonic LH release, whereas non-specific opioid antagonists, such as naloxone, increase LH release and block opioid-induced LH suppression in ewe (Knight *et al.*, 1990; Barb *et al.*, 1991; Currie *et al.*, 1991; Whisnant *et al.*, 1991). Naloxone can act at all the OR subtypes and can reverse the actions of opioid agonists (Hayes & Stewart, 1985; Conover *et al.*, 1993; Dooley *et al.*, 1998). The suppressive effects of opioids are mediated through regulation of hypothalamic GnRH secretion rather than directly on the gonadotrophs, as ORs are not present in the anterior pituitary. Also, opioid agonists do not reduce LH concentrations in hypothalamo-pituitary

disconnected ewes maintained on pulsatile GnRH (Horton *et al.*, 1990). Furthermore, in E_2-treated OVX ewes, effects of opioid antagonists on LH amplitude operate through regulation of GnRH secretion (Goodman *et al.*, 1995a). Clearly, opioids in the hypothalamus form important inhibitory pathways of neural circuitry that are involved in regulation of gonadotrophin secretion (Kalra, 1993).

Steroid influence on opioids

Within the hypothalamus, the exact pathway or the site of opioid regulation of GnRH secretion remains to be ascertained. Steroid-receptive β-endorphin cell bodies are localised mainly in the ARC region of the MBH in the rat and ewe (Fox *et al.*, 1990; Lehman & Karsch, 1993). Steroid treatments increase opioid gene expression in the ewe ARC (Whisnant *et al.*, 1992; Broad *et al.*, 1993). Also, during both luteal and follicular phases of cycle, opioid antagonist infusion into the ME close to the MBH increases GnRH concentrations, whereas β-endorphin infusion at the same level reduces GnRH concentration suggesting that opioids inhibit GnRH secretion in the vicinity of GnRH fibres and terminals in the MBH-ME (Conover *et al.*, 1993). After peripheral opioid antagonist administration in luteal phase ewes, a six-fold increase in Fos-active GnRH neurones located exclusively in the ovine MBH indicate the preferential inhibition of MBH GnRH neurones by opioids (Boukhliq *et al.*, 1999). Moreover, neural lesion studies show that even after anterior hypothalamic deafferentation, opioid antagonist injection increases LH pulse frequency in progesterone-treated ewe and progesterone suppresses LH pulse frequency in OVX ewe (Whisnant & Goodman, 1994). Thus, progesterone influence on GnRH neurones through an opioid neurones located in the MBH is sufficient and connection between POA and MBH is not required for suppression of GnRH pulse frequency during progesterone dominance. However, delay in the onset of an LH surge in E_2-treated OVX ewes by the bilateral infusion of a highly selective ORμ agonist, into either the mPOA or the MBH, suggests a dual hypothalamic site for opioid regulation of GnRH surge secretion (Walsh & Clarke, 1998). Also, administration of the β-endorphin or met-enkephalin in the POA or MBH, respectively, increases LH release in luteal phase ewes (Weesner & Malven, 1990). Similarly, opioid antagonist implants in either area increase LH pulse frequency. In the follicular phase, application of an opioid antagonist in

the POA or the MBH, respectively, increases LH pulse frequency or LH pulse amplitude (Whisnant *et al.*, 1991). These results suggest that although opioids act in both the POA and MBH to inhibit LH secretion, different populations of opioid neurones may be activated during different phases of the oestrous cycle.

Pathway of opioid-GnRH neuronal interaction

In potential support of dual regulation, various workers suggest an opioid neural link between the MBH and POA. In the rat, retrograde tracer studies show that β-endorphin and ACTH axon terminals from the ARC project to the medial preoptic nucleus and make synaptic contacts with neurones to the hypothalamic PVN (Kawano & Masuko, 2000). Also in ewe, terminals from the ARC β-endorphin neurones project to the POA (Whisnant *et al.*, 1992). About 40% cells projecting from the ewe MBH to the POA contain ERα (Goubillon *et al.*, 1999), but controversy exists for close contact between these projections and GnRH cells in the POA (Pompolo *et al.*, 2001; Goubillon *et al.*, 2002). Therefore, either direct or a local interneuronal network in the POA between the MBH neurones and GnRH perikarya transmits opioid effects from the MBH to the POA. Further, in ewe a delayed and transient effect of an OR agonist on plasma LH concentrations suggests that opioids influence GnRH cell bodies through interneurones (Walsh & Clarke, 1996). Noradrenaline, GABA, glutamate and/or nitric oxide-positive neurones may be potential mediators as opioids restrain stimulatory catecholamine, glutamate and nitric oxide input and accelerate inhibitory GABA input to GnRH neurones in the rat (Kalra, 1993; Yilmaz *et al.*, 1996; Bhat *et al.*, 1998; Faletti *et al.*, 1999). Also, in ewe, opioid antagonist injection suppresses preoptic GABA, suggesting opiate-GABA interaction but the absence of a change in preoptic noradrenaline release questions opiate-noradrenaline interactions in this species (Robinson & Kendrick, 1992a).

In fact, the likelihood of a direct influence of opioids on GnRH cell bodies and terminals also exists. In the ewe ME, β-endorphin can act to inhibit GnRH pulse frequency (Conover *et al.*, 1993), where endorphin neurones, projecting out of the MBH, are near GnRH terminals (Whisnant *et al.*, 1992). In the POA and the MBH, synaptic contacts between β-endorphin and GnRH neurones occur in the rat and monkey (Leranth *et al.*, 1988b; Thind & Goldsmith, 1988; Chen *et al.*, 1989a). Direct actions of ORμ

agonists on the hyperpolarisation of GnRH neurones in the guinea pig suggest that normal GnRH neurones may coexpress different types of ORs. Furthermore, functional ORδs mediate opioid inhibition of GnRH secretion in rat GnRH-producing cell lines, (Lagrange *et al.*, 1995; Maggi *et al.*, 1995). However, although ORs are found in many cells within the rat POA, ORμ, -δ or -κ mRNAs are not expressed in GnRH neurones, placing into question the direct influence of opioids on GnRH neurones (Mitchell *et al.*, 1997; Sannella & Petersen, 1997).

Changes in opioid tone during oestrous cycle

During the ewe oestrous cycle, inhibitory opioid tone increases from luteal to follicular stages as evidenced by increasing β-endorphin concentrations in the ME (Domanski *et al.*, 1991; Conover *et al.*, 1993). However, there is no change in POMC gene transcription between the luteal and follicular phase (Walsh & Clarke, 1998). Nevertheless, gradual increases in β-endorphin release without alteration in POMC mRNAs during the oestrous cycle may occur by post-transcriptional opioid transport and release (Skinner & Herbison, 1997). Furthermore, there is a two-fold increase in the number of ORμ and -δ in the MBH and POA, from the late luteal to the follicular phase in ewes (Thom *et al.*, 1996). Overall, a continuous inhibitory tone coupled with high receptor numbers in the pre-surge period may play a particularly important role in preventing the premature activation of GnRH neurones early in the follicular phase. Indeed, opioid tone may be involved in synchronising the activity of the GnRH neurones to determine the dynamics of individual GnRH pulses. There is evidence to support this: restraining the opioid influence with an opioid antagonist in OVX ewes rapidly prolongs GnRH pulses as well as increasing the inter-pulse interval (Goodman *et al.*, 1995a). Indirectly, opioid antagonists or agonists, respectively stimulate or inhibit MUA duration in the rat, monkey and goat (Williams *et al.*, 1990; Kimura *et al.*, 1991; Ito *et al.*, 1993).

In contrast to the continuous inhibitory opioid tone during the luteal and follicular phases, there is a substantial reduction in opioid activity 24 h before the LH surge as revealed by decrease in concentrations of β-endorphin in the ME (Domanski *et al.*, 1991). This reduction in the opioid brake (disinhibition) is permissive for the generation of the preovulatory GnRH/LH surge (Currie *et al.*, 1991; Kalra, 1993; Clarke, 1995a; Walsh &

Clarke, 1998). Consistent with this hypothesis, activation of ORμ by a specific receptor agonist delays the onset of E_2-induced LH surge in OVX ewes (Walsh & Clarke, 1996; Walsh & Clarke, 1998). However, the neural mechanisms of disinhibition are unlikely to be through OR suppression, as there is no marked reduction in OR number nor ORμ mRNA expression in the ewe hypothalamus around the time of the LH surge nor across the oestrous cycle (Thom *et al.*, 1996; Walsh *et al.*, 2001). Changes in POMC mRNA concentrations in the ARC are also not evident during the preovulatory period, whereas pro-enkephalin gene expression may be permissive for the surge, as pro-enkephalin mRNA concentrations are lower during the follicular phase and at the time of the LH surge in the ewe MBH (VMN) and PeVN (Walsh & Clarke, 1998; Walsh *et al.*, 2001). On the other hand, the stimulatory influence of E_2 to start the preovulatory surge seems to play an important role in the mechanism of pre-surge opioid disinhibition in the rat, by decreasing number of ORμ in the POA, POMC gene expression in the ARC and β-endorphin release (Wise *et al.*, 1990; Rosie *et al.*, 1992; Maggi *et al.*, 1993). Also, enkephalin cell types in the ARC that project to the POA coexpress ERs in the rat (Simerly *et al.*, 1988). However, in ewe, little is known about the precise mechanisms underlying E_2-induced changes in the opioid disinhibition during the presurge period.

Various workers have administered opioid agonists/antagonists in the pre-surge period to examine the effect on the timing of the LH surge. In women and rats, opioid agonists consistently block, and antagonists advance, the timing of the preovulatory GnRH/LH surge (Ching, 1983; Allen & Kalra, 1986; Allen *et al.*, 1988; Rossmanith *et al.*, 1988). In contrast in ewe, infusion of naloxone does not advance timing of the surge in intact ewes or after E_2-treatment in OVX ewes (Knight *et al.*, 1990). In other reports, however, long-term infusion of opioid agonists during the follicular phase delays or prevents the LH surge and blocks ovulation in gonad-intact cyclic ewes (Brooks *et al.*, 1986). The possible explanation for opioid antagonists not advancing the time of the LH surge in ewe could be that neural mechanisms to trigger the GnRH/LH surge include withdrawal of inhibitory tone plus input of excitatory tone. Therefore, in spite of removal of inhibitory opioid tone, there could be a lack of withdrawal of the GABA brake, and no acceleration of the stimulatory input of NPY or noradrenaline neurones for generation of the preovulatory LH surge (Kalra, 1993; Clarke, 1995a).

Evidences for the role of opioids in progesterone feedback

How is the well-known progesterone-induced inhibition of LH pulse frequency at hypothalamic level, mediated through opioids in ewe irrespective of season (Martin *et al.*, 1983; Karsch *et al.*, 1987)? In the rat, PRs have been well characterised in β-endorphin/enkephalin neurones (Olster & Blaustein, 1990), hence the possibility of PR colocalisation in ewe opioid neurones exists. The concept of a major opioid-mediated suppressive role for progesterone, and not E_2, is supported by studies showing that an opioid antagonist increases LH pulse frequency in progesterone-treated OVX ewes in anoestrus, in the breeding season and also in the luteal phase, but not in intact ewes or E_2-treated OVX ewes during anoestrus and in absence of progesterone during breeding season (Meyer & Goodman, 1985; Brooks *et al.*, 1986b; Horton *et al.*, 1987; Trout & Malven, 1987; Whisnant & Goodman, 1988; Yang *et al.*, 1988; Whisnant & Goodman, 1994; Boukhliq *et al.*, 1999). In the rat, opioids play an important role in mediating the negative feedback action of progesterone on GnRH/LH pulse frequency as high progesterone concentrations during the luteal phase elevate β-endorphin mRNA concentrations (Petersen *et al.*, 1993). Results of progesterone treatment alone on POMC mRNA concentration are not available in ewe, although progesterone in combination with E_2 or E_2 alone similarly increases POMC mRNA concentrations in the ARC (Whisnant *et al.*, 1992; Broad *et al.*, 1993). However, no influence in POMC mRNA expression in ewe hypothalamus between the luteal and follicular phases suggests that increased POMC gene expression is unlikely to be the mechanism of progesterone negative feedback (Walsh *et al.*, 1998). A high density of enkephalin-containing neurones is located in the PeVN (Broad *et al.*, 1993) and in this area at the time of GnRH/LH surge, large number of neurones express Fos suggesting that these neurones are involved in mechanisms underlying the surge (Moenter *et al.*, 1993). Whether these neurones are enkephalin-positive is not known. Progesterone decreases pro-enkephalin mRNA in the VMN region of the MBH in OVX ewes although E_2 treatment significantly increases pro-enkephalin mRNA expression (Broad *et al.*, 1993). Here, hormonal treatments are used to mimic concentrations of late pregnancy; thus, it is less convincing to correlate these results with oestrous cycle changes. Across the oestrous cycle, pro-enkephalin expression is

suppressed during the presurge period in the VMN, but it remains to be determined whether this is due to fall in progesterone or rise in E_2 or both (Walsh *et al.*, 2001). Lastly, it seems possible that inhibitory effects of progesterone during the luteal phase might involve enkephalin peptides because; first, an intracerebroventricular infusion of met-enkephalin antagonist in the MBH stimulates LH release (Weesner & Malven, 1990). Second, opioid cell population in the MBH is sufficient to generate suppressive action of progesterone (Whisnant & Goodman, 1994).

Opioid mediation of E_2 negative feedback during breeding season

By what mechanisms do opioids mediate E_2 feedback on GnRH neurones during the breeding season? In this season, E_2 does not have a dominant prolonged negative feedback effect on tonic LH secretion though E_2 does reduce LH pulse amplitude and stimulates LH pulse frequency. The increase in LH pulse frequency is due to the stimulatory effect of E_2 on GnRH pulse frequency. The E_2-induced decrease in LH pulse amplitude is the result of double action: by decreasing pituitary responsiveness to GnRH and by inhibiting GnRH pulse amplitude from the hypothalamus (Karsch *et al.*, 1983; Evans *et al.*, 1994; Goodman *et al.*, 1995a). In ewe, a subset of β-endorphin neurones in the MBH contains ERα (Herbison *et al.*, 1993b). Oestradiol treatment enhances POMC mRNA and pro-enkephalin mRNA concentrations in the MBH in OVX ewes (Whisnant *et al.*, 1992; Broad *et al.*, 1993). Interestingly, following acute E_2 challenge in OVX ewes, only 2% of the total number of Fos cells in the ARC were ACTH (POMC)-positive (Clarke *et al.*, 2001).

The lack of alteration in GnRH pulse frequency both in E_2-treated OVX and OVX ewes after opioid antagonist treatment shows that opioids are not involved in E_2-induced increase in LH pulse frequency (Goodman *et al.*, 1995a). The stimulatory effects of opioids antagonists on LH pulse amplitude and frequency in the follicular phase of the ewe oestrous cycle, and in E_2-treated OVX ewes, but not in OVX ewes, suggests that opioids mediate E_2- negative feedback on LH pulse amplitude. During follicular phase, opioid antagonists increase LH pulse amplitude and pulse frequency. The latter may be result of progesterone exposure as LH pulse frequency increases slightly in recently OVX ewes but not seen in chronically OVX ewes. However, no increase in LH pulse amplitude

is observed in any of the OVX ewes (Brooks *et al.*, 1986c; Currie & Rawlings, 1987; Trout & Malven, 1987; Whisnant & Goodman, 1988; Yang *et al.*, 1988; Horton *et al.*, 1989; Whisnant *et al.*, 1991). By contrast, an opioid antagonist-induced increase in GnRH pulse size, both in OVX ewes and E_2-treated OVX ewes, shows that although opioids inhibit GnRH pulse size, they do not mediate the negative feedback of E_2 during the breeding season (Goodman *et al.*, 1995a). Still, opioid mediation of E_2 negative feedback on ovine GnRH neurones in the breeding season cannot be ruled out. After the end of an opioid antagonist infusion, the increase in GnRH pulse size decreases abruptly in OVX ewes but remains high for many hours in E_2-treated OVX ewes (Goodman *et al.*, 1995a). Also, during ewe follicular phase, opioid antagonist infusion leads to increase in GnRH pulse amplitude in push-pull perfusates of the ME (Conover *et al.*, 1993). In conclusion, E_2-induced inhibition of GnRH pulse amplitude could be opioid-mediated but opioid influence on GnRH neurones may involve indirect pathways. One possibility is noradrenergic neurones as these appear to be involved in inhibition of LH pulse amplitude in ewes (Goodman *et al.*, 1996). Also, noradrenergic neurones mediate, at least in part, the suppressive effects of opiates on GnRH release in rat through ORs present on the noradrenergic terminals (Kalra & Kalra, 1984; Leadem & Kalra, 1985; Bouvier *et al.*, 1998; Yilmaz & Gilmore, 1999).

Alterations in OR dynamics

Depending on the steroidal environment during the oestrous cycle or after different steroid treatments of OVX ewes, region-specific receptor subtype alterations can occur in the hypothalamus of the ewe, without any change in total number of ORs (Shen *et al.*, 1995; Thom *et al.*, 1996). A consistent increase in ORμ and ORδ (Thom *et al.*, 1996), explains an opioid antagonist mediated increase in LH pulse frequency or amplitude during the follicular phase. Furthermore, an increase in ORδ in the POA in the luteal phase, or in progesterone-treated OVX ewes, over that found in mid-follicular phase, or E_2-treated OVX ewes (Thom *et al.*, 1996) suggests that ORδ mediates the inhibitory effect of progesterone on LH pulse frequency in the POA. In the MBH of OVX anoestrous ewes, progesterone or E_2-treatment increases ORδ maximum binding and decreases affinity although effects are more pronounced with E_2. Characteristics of ORμ

are not altered by treatment with either E_2 or progesterone in the MBH. In the POA, E_2 treatment has no affect on ORμ or ORδ binding/affinity, although progesterone leads to 20% decrease in ORμ content without any effect on ORδ characteristics (Shen *et al.*, 1995). The importance of changes in OR subtype characteristics require further study. The results of OR manipulations with the central infusion of ORμ agonist strongly suggest that μ-receptor activation mediates the inhibitory input to GnRH neurones both in the vicinity of GnRH perikarya (in the mPOA) and GnRH fibres and terminals (in the MBH/ME) (Walsh & Clarke, 1998). The specific ORμ agonist significantly reduces LH concentrations and delays the E_2-induced LH surge in OVX ewes but the specific ORδ agonist had no affect (Walsh & Clarke, 1996; Walsh & Clarke, 1998). Also, in the rat, the majority of evidence suggests that ORμ is responsible for mediating the inhibitory opioid brake on GnRH secretion as results are inconsistent for ORδ (Pfeiffer *et al.*, 1983; Herbison, 1995; Yilmaz *et al.*, 1996).

Furthermore, the physiological role of subtype ORκ in the control of GnRH/LH secretion is unclear, though ORκ density is higher than ORδ, in ewes. As the specific ORκ agonist has no effect on LH concentrations and κ-receptor characteristics are not altered by steroid treatments, it appears that ORκ are not involved in regulating GnRH secretion (Shen *et al.*, 1995; Walsh & Clarke, 1996). However, in another study, opioids were suggested to act via ORκ in the MBH to inhibit LH pulse frequency during the luteal phase in sheep (Goodman *et al.*, 1999). Clearly in the ewe, more studies using microinfusions of highly specific OR agonists at physiological dose levels and into specific sites in the hypothalamus are required to clarify the role of specific OR subtype

GnRH surge induction process and its interruption

In artificial cycles of OVX ewes, E_2 implants inserted 16 to 24 h after progesterone withdrawal, will generate follicular phase E_2 concentrations leading to a gonadotrophin surge 15-20 h after implant insertion (Moenter *et al.*, 1990; Evans *et al.*, 1997; Battaglia *et al.*, 1999; Harris *et al.*, 1999). During a natural preovulatory period,

elevated E_2 concentrations start decreasing about 4 h after GnRH surge onset and become basal within 12 h, although the GnRH surge persists for 18-24 h (Caraty *et al.*, 1989; Moenter *et al.*, 1990; Moenter *et al.*, 1991). This indicates that E_2 is required for GnRH surge initiation but not for surge maintenance. Experiments have varied the duration of this E_2 exposure in OVX ewes to identify the precise pattern of E_2 signal required for induction of the gonadotrophin surge. Removal of E_2 implants 2 h before the expected time of surge onset neither alters the LH surge in relation to that induced when the E_2 stimulus is maintained, nor affects stimulation of GnRH surge that outlasts the LH surge. However, continuation of the E_2 stimulus does prolong the GnRH surge by 10 h. With a full duration or abbreviated E_2 signal, there is no difference in GnRH surge amplitude although the LH surge is of reduced amplitude in the latter. This suggests that there is requirement for longer E_2 priming at the level of pituitary for a full amplitude LH surge (Evans *et al.*, 1997; Battaglia *et al.*, 1999). Oestradiol enhances pituitary responsiveness to GnRH by increasing GnRH receptor number/mRNA in gonadotrophs (Kaynard *et al.*, 1988; Gregg & Nett, 1989; Hamernik *et al.*, 1995; Brooks & McNeilly, 1996).

By experimentally shortening the E_2 signal during the presurge period, the approximate duration of the E_2 stimulus required for development of the GnRH surge is from 7 to 14 h (time zero is 16 h after progesterone withdrawal), well in advance of GnRH surge onset (Evans *et al.*, 1997). In later studies, this period of essential E_2 exposure is redefined as between 5-10 h (Harris *et al.*, 1999; Richter *et al.*, 2001a). The onset of the GnRH/LH surge occurs about 21 h after the start of the E_2 stimulus irrespective of both duration of the E_2 signal or the time of the stimulus relative to progesterone withdrawal (Evans *et al.*, 1997). This separation between the E_2 stimulus and surge generation, along with the absence of ERs within GnRH neurones (Lehman *et al.*, 1993; Lehman & Karsch, 1993) indicates; first, transmission of E_2 information to GnRH neurones occurs either directly via E_2-receptive neurones or through at least one set of interneurones interposed between E_2-receptive neurones and GnRH neurones. Second, E_2 is required only for activation of the E_2-responsive neurones and not for transmission of the stimulatory signals from these neurones to GnRH neurones responsible for the actual surge release of GnRH (Evans *et al.*, 1997). In addition, a 6 h

delay in the negative feedback effects of E_2 on GnRH secretion, further suggests an indirect route of E_2 action on the GnRH neurosecretory system (Evans *et al.*, 1994).

To elucidate the steps by which E_2 induces the gonadotrophin surge, a surge induction process temporally divided into three stages has been hypothesised (Evans *et al.*, 1997; Battaglia *et al.*, 1999; Harris *et al.*, 1999). Stage I of this model is E_2-dependent whereas stages II and III are oestradiol-independent. Starting from E_2 implant insertion, the first stage is an E_2 signal-reading or activation stage (from 5 to 10 h), during which the E_2 signal for the preovulatory surge activates E_2-sensitive neurones. The second stage is for signal processing or transmission (from 11 to 16 h), when the signal is processed and transmitted to GnRH neurones by E_2-responsive neurones or via one or more interneurones. The third stage involves onset of surge release (beginning at 21 h), the time of actual discharge of GnRH that stimulates the LH surge.

This model for the surge-induction process in the ewe can provide insight into the mode of action of various surge-disrupters as well as explanations for the suppressive effects of progesterone on the gonadotrophin surge. Systemic challenge with an immune stressor, such as endotoxin, hinders the normal progression of the follicular phase and suppresses both tonic GnRH and LH release (Battaglia *et al.*, 1997; Battaglia *et al.*, 2000). During an artificial follicular phase (E_2 signal for 48 h), endotoxin infusion (from 0 to 30 h) delays the onset of the LH surge, without any effect on surge amplitude, duration or incidence. A possible reason for this regeneration of the LH surge is the development of tolerance to immune challenge, as indicated by the decline of adrenal steroid concentrations (progesterone and cortisol) and return of body temperature to pre-treatment values by the end of endotoxin infusion. In contrast, the LH surge is blocked when the E_2 stimulus is removed at the end of endotoxin infusion (for 12 or 14 h) suggesting that after removal of endotoxins, but without a continued E_2 stimulus, the GnRH neurosecretory system is unable to restart the surge process. Thus, endotoxins appear to interfere with the surge induction process during activation of the E_2-responsive neuronal population, but have no effect on events occurring during signal transmission or during surge release (Battaglia *et al.*, 1999). In a similar manner, ethanol delays the LH surge when present during start of E_2 signal in OVX ewes (Goebbert *et al.*, 1996). Transport of E_2-treated ewes just before the LH surge delays surge onset suggesting

disruption of the later phase of the transmission pathway by transport (Smart *et al.*, 1994). However, insulin stress in ewes disrupts the surge at any time before actual release of LH (Medina *et al.*, 1998).

Progesterone inhibits tonic LH release during the luteal phase and blocks the LH surge (Karsch *et al.*, 1979). Under high progesterone concentrations in the luteal phase, E_2 is unable to stimulate the hypothalamic surge generating mechanism, despite the presence of follicular phase E_2 concentrations (Hauger *et al.*, 1977; Currie *et al.*, 1993). The suppressive action of progesterone is at the level of the hypothalamus and not the pituitary because GnRH surge is suppressed, but exogenous GnRH can stimulate LH release in ewes treated with progesterone (Kasa-Vubu *et al.*, 1992; Harris *et al.*, 1999).

Recent studies demonstrate the ability of progesterone to block the surge-generating E_2 signal during the activation stage or during the initial phase (10-15 h) of signal transmission. During the later period of signal-transmission, immediately before the onset of the surge, progesterone is unable to block the GnRH surge, indicating that progesterone does not act directly on GnRH neurones (Harris *et al.*, 1999; Richter *et al.*, 2001a). In support, PRs are not found on GnRH neurones (Skinner *et al.*, 2001). The interneuronal systems that mediate progesterone negative feedback are thought to be not independent of neural pathways that mediate E_2 positive feedback. In fact, progesterone treatment prevents the activation of both GnRH and non-GnRH neurones associated with the E_2-induced surge (Richter *et al.*, 2001a). Whether these non-GnRH cells are E_2-receptive is still to be determined by Fos and immunohistochemical double-labelling studies. In conclusion, progesterone may act directly upon E_2-responsive neurones to prevent activation by E_2, or the progesterone-responsive elements that impinge upon E_2 responsive neurones mediate suppressive effects by disrupting the activation of pathways essential for early phase of transmission of surge-inducing E_2 signal (Harris *et al.*, 1999; Richter *et al.*, 2001a).

Inhibition of noradrenergic or activation of opioid and GABA pathways by progesterone during the regulation of tonic gonadotrophin release has already been discussed in this review. The inability of progesterone to suppress LH pulse frequency in naloxone-treated ewes indicates an opioid action of progesterone on tonic LH release. In contrast, naloxone is unable to restore an E_2-induced LH surge blocked by progesterone

treatment during the activation stage of GnRH surge induction. However, naloxone restored LH surge (of reduced magnitude) in progesterone-treated ewes during the transmission stage. In conclusion, progesterone does not block the activation of E_2-responsive positive feedback systems via opioids though opioids appear to be part of the neural pathways by which progesterone disrupts the transmission phase (Richter *et al.*, 2001).

Activation of stress pathways and regulation of GnRH release

Two publications with up to date knowledge of the interrelationships between many brain structures involved in stress modulation of reproduction have been prepared and published during preparation of this thesis. Copies are attached near the end of this submission.

Summary

Within GnRH perikarya, classic interplay between ovarian steroids dictates the timing of the appropriate GnRH release into the pituitary portal circulation. During the luteal phase, E_2 plays a modest suppressive role by decreasing GnRH pulse amplitude while progesterone has a dominant suppressive effect on GnRH pulse frequency. In contrast, after luteolysis, the decline in progesterone concentrations allows the positive feedback of E_2 to elevate GnRH pulse frequency leading to a preovulatory surge. For a full GnRH surge, progesterone pre-treatment induces an increase in ERs and thus facilitates the effects of E_2 within the ER-expressing neuronal component of the surge-generating network. Considerable efforts have been made to understand the precise mechanism and neuronal pathway of steroid signalling and transmission within the hypothalamus. The most likely hypothalamic sites of action are the mPOA and MBH (VMN and ARC), containing 60% and 15% of GnRH cell bodies, respectively as well as a high density of steroid receptors, and/or the ME, where GnRH axons terminate on hypophyseal portal blood vessels. In the mPOA and MBH, PR positive cells also have

ERα, however only ERs are found in the brainstem, an extra hypothalamic site of steroid action.

For genomic influence, steroids do not act directly on GnRH neurones because; first, there is little evidence for the presence of classical steroid receptors within GnRH neurones. Second, steroid implants placed into the mPOA do not affect GnRH release although the mPOA contains the highest density of GnRH perikarya. Therefore, the prevailing hypothesis is that steroids modulate GnRH neuronal activity indirectly via a mediating system comprising a single interneurone or a myriad of interneurones. Oestradiol acts on GnRH neurones through interneurones colocalising ERs that are noradrenaline (in the BS), opioids (in the ARC) and GABA (in the POA). The precise chemical identity of progesterone-receptive interneurones is currently not known in the ovine hypothalamus; however, progesterone exerts suppressive effects on GnRH neurones by modulating the release of noradrenaline, opioids and GABA. Additional proof for neuromodulators mediating steroid influence on GnRH neurones is provided by the presence of numerous neurotransmiter synaptic inputs on GnRH neurones. In the ewe POA, GABA neurones form synaptic contacts with GnRH cell bodies. Furthermore, noradrenaline and β-endorphin terminals form an intricate network in close apposition to GnRH cell bodies. At the level of the ME, extensive noradrenergic innervations are present. Enkephalin and β-endorphin-containing fibres are also found in the ME.

The surge induction model in OVX ewes comprising of signal activation, transmission and surge-release stages has been used to clarify the indirect route of steroid action on GnRH neurones. This model suggests; first, the E_2 signal for activation of the E_2-responsive elements is of short duration and ends well in advance of GnRH surge onset. Second, a direct action of E_2 on GnRH neurones is not involved at the time of the surge. Third, the ability of progesterone to block interneuronal systems from reading E_2 signal and to block early, but not later, part of signal transmission indicates indirect action of progesterone on GnRH neurones, either via E_2-responsive cells activated by E_2 or through progesterone-responsive cells that impinge upon E_2-responsive cells. A number of studies have been done to understand the mechanism of surge induction in OVX ewe model but nothing is known about the duration of three-stages of surge-induction model in intact animals. Whether the time frame for activation of neuroendocrine systems in

intact and OVX ewes is different in response to endogenous and exogenous steroid signal, respectively, remains to be investigated.

Attempts have been made to delineate the neuroanatomical loci and the pathways through which steroids exert feedback actions on GnRH neurones. Two steroid-responsive neuronal circuits located in intrinsically different hypothalamic compartments, which are, however, difficult to characterise, control tonic and surge gonadotrophin release. Electrophysiological correlates demonstrate the MBH as a site for GnRH pulse generation though these studies are unable to elucidate surge generation site. Deafferentation and Fos data suggest that a population of GnRH neurones in the MBH is responsible for tonic release and the preovulatory surge is the result of contributing from rostrally (mPOA) as well as caudally (MBH) placed GnRH neurones. Immunohistochemical and implant insertion studies implicate the MBH, but not the mPOA, as a key area for steroid interaction. First, there is a large density of ER and PR positive cells that undergo changes during the oestrous cycle in the MBH but not in other hypothalamic regions. Second, increases in ERs in the VMN or PRs in the VMN and ARC occur after progesterone or E_2 treatment, respectively. Third, GnRH surge onset or blockade is seen after placing E_2 or progesterone implant, respectively, in the VMN but not in the mPOA.

Collectively, to evoke GnRH surge, principal site of E_2 action, the VMN, is remote from the mPOA. The possibility remains that E_2 elicit feedback signals via multiple routes to regulate preoptic GnRH neurones. First route: Following E_2 treatment, Fos is expressed within 1 h in the MBH. After activation of E_2 responsive cells in the MBH, the signal may be transmitted to the mPOA. However, neuronal communication between these hypothalamic sites is yet to be fully investigated. Anterograde neuronal tracing recently reveals conflicting reports regarding neuroanatomical connections between the VMN and the mPOA. Thus it is not clear whether there is direct and/or indirect (via local interneuronal network in the POA) regulation of preoptic GnRH neurones by VMN projections. Within these projections, a subset of E_2-receptive input from the VMN to the POA may represent the route through which E_2 coordinates activity of neuromodulators associated with surge-induction. Unfortunately, the neurochemical identity of the majority of E_2-responsive cell types in the MBH that regulate GnRH

neurone activity remains enigmatic because after acute E_2 challenge only 30% of the total Fos-positive cells are ACTH, NPY and TH-positive in the ARC, and none of the somatostatin-positive cells in the VMN is Fos-positive. However, others found Fos positive somatostatin neurones following E_2. The majority of ER-containing cells in the VMN express somatostatin but the relationship with GnRH neurones is not known.

Second route: This E_2-triggered surge-generating route might start from the MBH going through the brainstem to GnRH neurones in the mPOA. After E_2 treatment, Fos is observed within 2 h in the brainstem VLM noradrenergic cell group followed by time-delayed increase in noradrenergic activity (maximum around 8 h) or loss of DβH-immunostaining (at 16 h) in the POA, which may be related to E_2 positive feedback. But, conflicting results are present regarding increased noradrenergic activity in the MBH-ME region at the time of E_2-induced surge. Apparently, E_2 activation signal may directly start from the brainstem noradrenergic neurones or through the descending afferents (neurochemical identity unknown) from the VMN to the brainstem. Undertaking a series of experimental approaches can conclusively test this. First, inserting an E_2 implant in the VMN along with the Fos studies in the VLM. Second, surge generation after placing an E_2 implant in the VLM. Third, abolishing GnRH surge with blockade of adrenoreceptors in either or both of these regions during signal-activation stage. From the brainstem, the signal may be transmitted to the mPOA via ERα-positive cells projecting from the caudal part of VLM to the mPOA. In the POA, noradrenaline may directly act on GnRH neurones or via GABA neurones, which receive synaptic input from noradrenaline and then synapse on to GnRH neurones.

Third route: Oestradiol may directly act on GnRH terminals located in the external zone of ME through the neuronal link between the MBH and the ME. But, this connection has a little role, if any, in the onset of surge because the ME receives a very small proportion of ER-containing projections and anterograde-labelled fibres from the ARC and VMN, respectively. Lastly, in contrast to the MBH as a primary area for E_2 positive feedback, it is not possible to point towards a single hypothalamic area involved in suppressive action of E_2.

The nature of the signalling pathways by which steroids interact with neurotransmitters, to control tonic and surge release of gonadotrophin, is under intensive

investigation. During stages of tonic gonadotrophin secretion, under progesterone dominance, inhibitory route is active through suppression of noradrenergic neurones and stimulation of opioids and GABA neurones. Here, the action of progesterone on GABA systems is mediated by opioid neurones through its classic nuclear PRs and a direct interaction of progesterone metabolite (allopregnanolone) with $GABA_A$ receptor is ruled out. How progesterone suppresses the noradrenaline network is unknown. There is little evidence for the involvement of E_2-receptive interneuronal systems in the inhibitory action of E_2 on tonic GnRH release. The role of opioids is controversial. One possible mediator is noradrenaline neurones as α-adrenoreceptor antagonists (in the POA) increases GnRH pulse amplitude inhibited by E_2, though further work is needed to adequately test this hypothesis.

During presurge period, a short duration of surge producing E_2 stimulus follows a prolonged delay in surge onset. A fundamental question is on which interneuronal cell population does E_2 act to initiate activation signal. The noradrenergic neurones, an important stimulatory component of the preovulatory surge mechanism, can be presented as probable candidates. But, in E_2-treated OVX ewes, no alteration in GnRH or LH pulse frequency after α-adrenoreceptor antagonist administration puts question. After signal reading, prolong signal-processing period corresponds to upstream regulation of sequential steps involving communication between multiplicity of neurotransmitters within GnRH network to generate surge-stimulating signal. It can be speculated that with E_2 trigger/dominance, besides the increase in noradrenergic activity, elevated opioids and GABA concentrations prevent the premature activation of GnRH neurones. However, immediately before the surge onset, suppression of opioids and GABA neurones facilitates noradrenaline neurones causing switch from tonic to the surge release. Whether the disinhibition of opioid and GABA tone, at the end of presurge period, is under the effect of progesterone or E_2 or both is less clear. Interaction between noradrenaline and GABA systems during follicular phase in ewe remains to be determined to fully delineate the complexity of surge generating mechanisms. In the rat, noradrenaline stimulates GABA during E_2 negative feedback but during positive feedback in the preovulatory period, there is an uncoupling of noradrenergic stimulatory influence upon GABA neurones. Opioid inhibition of noradrenaline input to POA has a major control over

GnRH release in the rat. Whether noradrenaline component mediates the inhibitory effect of opioid pathway is less clear in ewe. Is the removal of opioid tone lead to the permissive nature of noradrenaline system within the surge-inducing network? How neurotransmitters modulate GnRH secretion at the level of the ME is another important area?

Progesterone and various other stress linked surge disrupters can delay or block the surge onset by influencing different stages of surge-induction process, however, underlying mechanism and the identity of interneuronal population or the pathways through which this effect occurs is not fully understood. In contrast to progesterone-induced inhibition of tonic GnRH release through opioid neurotransmission; during surge-release, opioid neurones are not the only mediators but may involve a range of other non-opioid neuronal systems. Whether progesterone blocks E_2-induced surge through rise in GABA concentrations or via suppression of noradrenergic neurotransmission is still to be validated. It is not clear which signal pathway activated by physical, metabolic or immunological stressors have the potential to disrupt normal onset of gonadotrophin surge.

Ultimately, does stress-induced decrease in gonadotrophin pulse frequency and/or amplitude during breeding season work through similar pathways activated during stress of long photoperiod. Do inhibitory signals override the stimulation of GnRH during early follicular phase? Nonetheless, it is beneficial to understand how and which neural components of surge-generating network do surge disrupters modulate. This will help to improve treatments for optimisation of fertility in various reproductive dysfunctions associated with stress and infection.

Objectives of this study

Functional and neuroanatomical studies were undertaken in this thesis to answer the following questions:

1. Does oestradiol (E_2) directly regulate hypothalamic AVP and GnRH release in ewes?
2. Does GABA have direct control of AVP release from the ewe hypothalamus and is this influenced by E_2?
3. What is the direct effect of E_2 on noradrenergic control of AVP release from the hypothalamus?
4. How does the GABA system regulate GnRH release within hypothalamus and does control of GABA depends on the E_2 milieu?
5. What is the correlation between the noradrenergic and GnRH systems within the hypothalamus under the influence of E_2?
6. What is the neuroanatomical relationship of noradrenergic and GABA systems with CRH, AVP and β-endorphin neurones within the hypothalamus?
7. Is there any interaction between CRH, AVP or β-endorphin and GnRH systems within the hypothalamus?
8. What mechanisms in the brainstem and hypothalamus are involved in stress-induced GnRH surge disruption during follicular phase?

Chapter 2

General materials and methods

General materials and methods

In vitro perifusion procedures

Media

Tissue was collected into Hank's Balanced Salt Solution without calcium and magnesium (pH 7.4, CMF-HBSS; Invitrogen, Paisley, Scotland), supplemented with 25 mM N-2-hydroxyethylpiperazine-N′-2 ethanesulfonic acid (HEPES, VWR, Poole, UK), sodium bicarbonate (0.35g/l; Sigma-Aldrich, Poole, UK), penicillin (10 units/ml; Sigma-Aldrich), and streptomycin (10μg/ml; Sigma-Aldrich). Transportation of tissue and perifusion was performed using Minimum Essential Medium-α (pH 7.4, MEM-α; Invitrogen), supplemented with 50 mM HEPES, 2.2g/l sodium bicarbonate, 0.1% bovine serum albumin (Sigma-Aldrich), 0.1% ascorbic acid (Sigma-Aldrich), 10 units/ml penicillin, 10μg/ml streptomycin, and 0.25μg/l fungizone (Invitrogen). The collection and perifusion media were prepared on the day of experiment and sterilized by filtration through a 0.22 μm Sterivex®-GS filter (Millipore, Billerica, USA). The media were continuously gassed with 95% O_2/5% CO_2 before and during use.

Hypothalamic slice and anterior pituitary tissue preparation

In the breeding season, brains were collected from ewes at a local slaughter house and the ovarian status was noted. Ten minutes after ewe sacrifice, whole brains were removed from the cranial cavity and placed in ice-cold oxygenated CMF-HBSS for 5 min. Brains were sectioned mid-sagittally. Hypothalamic blocks were obtained by free-hand cuts, using a skin graft surgical blade (Rocket Medical Plc, Washington, UK), to approximately 3 mm depth on the medial aspect of each hemi-hypothalamus at the following boundaries: coronal cuts through rostral edge of mammillary bodies and 4-mm rostral to anterior commissure, and a horizontal cut through the mid thalamus (Fig 2.1). A custom-built tissue slicer (McMahon *et al.*, 2001a) was used to prepare the final block at a distance of 2 mm parallel to the midline as follows: the medial aspect of each hemi-hypothalamus was placed against a vertical 5 x 7 cm block of glass. The section thickness was achieved by using two 1 mm thick glass slides,

glued to each side to create a vertical 2 mm gap. A single slice in the rostro-caudal direction provided a 2 mm saggital slice from each hemi-hypothalamus (Fig 2.1). The dimensions of such slices result in collection of an intact GnRH neuronal system (from the anterior preoptic area to the mediobasal hypothalamus with the median eminence, Fig 2.1A) along with the paraventricular and supraoptic nuclei (SON) containing AVP neurones within each hemi-hypothalamus slice. After collecting a minimum of 12 slices (2/sheep) in 30-40 min, single slices were transported to the laboratory within 40 min in glass vials containing ice-cold oxygenated MEM-α with or without E_2 (Sigma-Aldrich). For studies in chapter 7 and 8, pituitaries were also collected at the same time from the same ewes. With a surgical blade, anterior pituitaries were cut into 2 mm fragments and transported in a glass vial containing ice-cold oxygenated MEM-α without E_2.

Perifusion System

Within two hour of ewe sacrifice, each hypothalamic slice was transferred to the perifusion system comprising twelve in-parallel 25 mm perifusion chambers (Swinnex, Millipore) placed in lattice locations in water bath maintained at 37°C (Fig 2.2). Medium was drawn through the chambers by peristaltic pumps (Gilson minipulse-3, Villiers le Bel, France) at a rate of 0.15 ml/min and was gassed constantly with 95% O_2/5% CO_2 for at least three hours prior to and for the duration (8 h) of the experiment. The perifusion tubing (Internal diameter-1.01 mm, VWR) was connected to the bottom of the perifusion chambers so that medium flowed into the bottom of each chamber and out through the top. Three-way stopcocks were placed between the bottle containing the medium and the perifusion chambers so slices could be perifused with test compounds drawn from 2 ml syringes. To avoid any artefactual release of AVP, GnRH or LH, the test compounds were dissolved in perifusion medium obtained immediately before the time of exposure, from the bottle containing medium being used for perifusion during the experiment. The time taken by medium to flow from the three-way tap to the effluent collecting tubes was 20 min.

The perifusion assembly was modified in the experiments where anterior pituitary fragments were perifused alone or in series with hypothalamic slices (Fig

2.3); however, medium flow rate and all other perifusion conditions remained the same. For the modification, another set of small size perifusion chambers (Swinnex 12 mm filter holder, Millipore), containing five pituitary fragments, were placed in series with a hypothalamic slice containing- or empty-chamber so that the hypothalamic perifusate or medium passed through the pituitary-containing chambers before fraction collection.

Following a four or five hour equilibration period to attain basal AVP, GnRH and LH release, 10 min serial effluent fractions (1.5 ml) were collected for three or four hours into tubes (on ice) containing 0.5 ml bacitracin (3x 10^{-3} M, Sigma-Aldrich) as a peptidase inhibitor. Samples were immediately prepared for AVP and LH assay and GnRH extraction. At the end of every perifusion, the system was flushed with 70% alcohol for two hours followed by sterile double distilled water to prevent microbial or fungal growth.

Treatments with low or high E_2 concentrations

Hypothalamic slices were perifused with medium containing low (6 pg/ml) or high (24 pg/ml) concentrations of E_2 (Phogat *et al.*, 1997). The low and high doses of E_2 used were three times the plasma values observed during luteal and follicular phase in ewes, respectively (Hauger *et al.*, 1977). These higher concentrations were chosen because E_2 concentrations in hypothalamus are above plasma values (Eisenfeld & Axelrod, 1965). Furthermore, the effect of these E_2 concentrations is within the physiological range (Phogat *et al.*, 1997). The appropriate dose of E_2 was solubilised first in absolute ethanol then perifusion media. Medium perifused without E_2 but containing appropriate ethanol concentrations served as a vehicle control. In each perifusion experiment, left and right side hypothalamic slices were distributed between groups and at least four slices were perifused per treatment.

Treatments with GABA $_{A\ or\ B}$ or α_1-adrenoreceptor agonist or antagonist (with high E_2 or without E_2)

After 60 min effluent collection, each slice with or without anterior pituitary tissue was treated for 10 min with vehicle (MEM-α), or a range of doses (0.1-10 mM)

of GABA $_{A \text{ or } B}$ or α_1-adrenoreceptor agonist or antagonist (with high E_2 or without E_2) by turning a 3-way stopcock to apply agonist/antagonist held in 2 ml syringes.

Hypothalamic slice and anterior pituitary tissue viability

One hour before the end of effluent collection, slices and pituitary tissue were exposed for 10 min to KCl (100 mM in media) to induce secretion of AVP, GnRH and LH. The period for which KCl was added is indicated in the appropriate figures by a yellow bar. Initially, after perifusion, six slices were fixed with paraformaldehyde (4%) overnight, and embedded in paraffin. Two saggital sections (5 μm) taken from the ventricular surface and in the middle of the 2 mm thick slice were stained with cresyl violet to check the integrity of the neuronal cell bodies.

Radioimmunoassay

AVP

AVP was assayed in 0.05 M phosphate buffer (pH 7.4) containing 0.1 M sodium chloride, 0.1% bovine serum albumin, 0.1% bovine gamma globulin and 0.1% sodium azide. Synthetic AVP (Sigma-Aldrich) was used for iodination, standards and quality controls. Duplicates of 50 μl effluent fractions were incubated at 4°C for 24 h with 50 μl 1:100,000 specific AVP antisera (code 4S12, raised in sheep, a gift of Dr S.G. Matthews and Mr C Chapman, BBSRC Babraham, Cambridge, U.K.). An aliquot of 50 μl 6,000 dpm iodinated AVP (iodinated in-house using NaI^{125}; Amersham, Luton, UK)(Moore *et al.*, 1977), was added and incubated at 4°C for a further 24 h. Bound hormone was precipitated by addition of 250 μl chilled 30% polyethylene glycol 8000 in assay buffer. After immediate centrifugation (30 min at 3500g, 4°C), the fluid aspirated, the pellet washed with 500 μl chilled aqueous 30% polyethylene glycol 8000, re-centrifuged, re-aspirated, and the precipitate was counted. None of the test substances interacted with the assay.

GnRH

Extraction: Immediately after collection of the effluent fractions (1.5 ml perfusate and 0.5 ml bacitracin), 3 ml methanol was added to 1.5 ml volume of the effluent. After centrifugation (30 min at 2500g, 4°C), the supernatant was decanted into clean glass tubes and dried overnight in a vortex evaporator (Savant SpeedVac plus concentrator, Holbrook, NY) at 40°C.

Assay: All solutions were prepared in 0.1 M phosphate buffer saline (pH 7.4) containing, 0.1% gelatin, and 0.1% sodium azide. Synthetic GnRH (Peninsula, St Helens, UK) was used for iodination, standards and quality controls. Dried extracts were resuspended with 225 µl assay buffer. Duplicates of 100 µl were incubated at 4°C for 24 h with 100 µl 1:4,000 specific GnRH antisera (Narayana & Dobson, 1979b). An aliquot of 100 µl 15,000 dpm iodinated GnRH (iodinated in-house using NaI^{125}; Amersham)(Caraty *et al.*, 1987), was added and incubated at 4°C for a further 24 h. Bound hormone was precipitated by addition of 2 ml ethanol (4°C) and after immediate centrifugation (30 min at 2500g, 4°C), the supernatant poured off, and the pellet counted. There was no interaction in the GnRH assay with E_2 or KCl. However, GABA and α_1-adrenoreceptor compounds interacted with the GnRH assay. This is further described in chapter 7 and 8. The results were corrected for percentage GnRH recovery and dilution.

LH

Concentrations of the LH in plasma or effluent fractions (4 µl fraction diluted to 100 µl with assay buffer) were measured in duplicates using 100 µl samples by the method characterised and verified in this laboratory (Dobson *et al.*, 1999). In the present study, anti ovine LH serum (NIDDK-anti-oLH, AFP-192279; 1:300,000 dilution) and iodinated oLH (NIDDK-oLH, AFP 7071B, 15000 cpm per 100 µl) were used, and results have been expressed as ng equivalents of NIH-LH-S21/ml plasma. None of the test substances interacted with the assay.

Cortisol

Total plasma cortisol concentrations were measured using 7 µl plasma, by the modified methods characterized and verified in this laboratory (Alam *et al.*, 1986). Briefly, plasma samples were treated with 1.0 ml double-distilled ethanol and the contents centrifuged for 10 min at 3000g. Following evaporation of supernatant, the extract was incubated at 4°C with 0.1 ml antiserum (1:2500 dilution) and ^{3}H-cortisol (10,000 cpm/0.1 ml), both in 0.1 M PBS gelatin. The separation of free hormone from the bound fraction was achieved by adding 2.5 ml scintillation fluid (Optiphase, Wallac, Milton keynes, UK), mixing and freezing the aqueous phase in an alcohol cooling bath, the organic phase was then decanted into scintillation vials and counted. The results were corrected for percentage GnRH recovery and dilution.

Glucose

Plasma glucose concentrations were measured by Mr P. Taylor, Small Animal Hospital, University of Liverpool, using the hexokinase/glucose-6-phosphate dehydrogenase method (Schmidt 1961). All reagents were from Boehringer Mannheim (Diagnostics and biochemicals) Lewes, Sussex, UK and the reaction was assessed in a Kem-o-mat 2 autoanalyser (Coulter Electronics, Luton, UK).

AVP, GnRH, LH, Cortisol and glucose assay characteristics

Sensitivity of the assays and inter- and intra-assay coefficients of variation are described in the respective chapters.

Section concerned with data analysis

Within group responses were analysed by comparing pre-compound control values with peak responses to compound addition. Comparison between groups used peak values of hormone responses without consideration of changes in basal release concentrations.

Immunohistochemistry procedures

Tissue collection

Brain samples were obtained from adult ewes during the breeding season. Ewes were sacrificed with intravenous sodium pentobarbitone (Pentobarbital, Loveridge, Southampton, UK) containing 25 000 IU heparin (Multihep, 5 ml 5000 IU/ml, Leo Laboratories, Princes Risborough, UK). The heads were immediately perfused bilaterally via the carotid arteries with 2 litres 0.9% saline containing 25 000 IU/l of heparin then 1.5 litres Zamboni's fixative (4% paraformaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4), followed by 500 ml of the same fixative containing 30% sucrose. These solutions were pumped through the brain with a peristaltic pump (Gilson's Minipuls-3, Villiers, France) and procedures from ewe sacrifice to end of perfusion were completed within 20 min. Brainstem and hypothalamic blocks were removed (Fig. 2.4), postfixed for 1.5 h in the fixative with 30% sucrose and finally left in 0.1 M PB containing 40% sucrose for 2 days at 4°C. The blocks were stored at -20°C, until 50 μm (brainstem, Fig 2.5) or 40 μm (hypothalamus, Fig 2.6) coronal sections were cut on a freezing microtome. All free-floating sections were left overnight in 0.1 M phosphate buffer saline (PBS, pH 7.4) before processing for immunohistochemistry.

Antisera

Antisera were used as detailed in Table 2.1. Controls were routinely performed by omitting primary antibodies which resulted in complete loss of staining in each case. In addition, higher dilutions of all these antibodies consistently reduced the intensity of staining.

Staining procedures

Details of the number of sections processed for dual-label immunohistochemistry using the free-floating technique are mentioned in chapters 9 and 10. Section washings, wherever mentioned, was with 0.1 M PBS, repeated four times for 10 min each time. Sections were initially washed and then incubated in 0.1% sodium borohydride (Sigma-Aldrich) for 20 min to remove excess aldehydes and washed again in PBS. This was followed by 2 h incubation in blocking solution containing 0.03% Triton-X (Sigma-Aldrich) and 10% normal donkey serum (Jackson

Immunoresearch, West Grove, PA, USA). Sections were double-labelled with the various combinations of antisera either with two antisera together or sequentially. Sequential staining was used where both Fos and other primary antibodies were raised in the rabbit. Due to the nuclear location of Fos, colocalisation with a cytoplasmic marker is possible (Doyle & Hunt, 1999).

During combined staining (all combinations of antisera in chapter 9 and 10 except those mentioned below for sequential staining), both antisera were diluted in PBS containing 2.5% normal donkey serum, 0.3% Triton-X and 0.25% sodium azide (Sigma) and incubated at 4°C for 72 h. On the fourth day, sections were washed in PBS and then incubated for 3 h at room temperature with a secondary antisera cocktail (1:100; donkey anti-rabbit Cy_3 or FITC, donkey anti-mouse Cy_3 or FITC; Jackson Immunoresearch) depending upon the primary antisera used. Thereafter, all sections were washed with PBS followed by a final wash with double distilled water.

During sequential staining with each antisera (Fos/CRH; Fos/AVP and Fos/β-endorphin), sections were first incubated with Fos antiserum and on fourth day treated with anti-rabbit Cy_3 (1:100). Thereafter, on the same day sections were incubated with CRH, AVP or β-endorphin antisera and on the seventh day exposed to anti-rabbit FITC (1:100). All the relevant incubations and washings were carried out using procedures outlined above. All sections were mounted on CAG (Chrom-Alum-Gel)-coated slides, dried and cover-slip applied using antifade medium (Vectshield, Vector Laboratories, Peterborough, UK).

Light microscopy

Sections were examined and image acquisition was made using a Zeiss axioskop microscope equipped with a Hamamatsu Orca ER digital camera and a PC equipped with image acquisition software (AQM, Kinetic imaging). Identification of double-fluorescent neurones (Fos or ERα with DβH or CRH or AVP or β-endorphin or GnRH) was achieved by switching from one filter cube to the other during the observation and single- or double-labelled neurones were recorded simultaneously.

Laser Scanning Confocal Microscopy (LSCM)

A Zeiss LSCM 510 microscope was used to examine the double-labelled immunofluorescent samples for close contacts between cell bodies and terminals. Sections were scanned with a 543 nm Helium-Neon filter to excite Cy_3 (red) fluorescence which was collected through a 545 dichroic mirror (DM) and 560 nm long pass filter. The 488 nm line from an argon ion laser was used to excite FITC (green) fluorescence which was collected from the 545 DM through a 505-530 nm band pass filter. Cross-talk between fluorochromes was eliminated by using 'tracking mode' where only the correct laser line is detected and each line of the images was scanned sequentially. Each section was scanned along the z-axis at 0.46 μm intervals of tissue section, at a resolution of 512 x 512 pixels; except for the ME, where resolution was 1024 x 1024 pixels. Possible interneuronal close contacts were examined in the parvocellular PVN, ARC, mPOA and in the ME. Close contacts were assessed by examining each of the 0.46 μm optical sections through the neurone cell bodies. At this resolution, it was possible to establish a close contact and to rule out a terminal/fibre presence over or under the neurone cell body.

Table 2.1 Primary antisera used to identify neurone cell bodies or terminals containing Fos, ERα, noradrenaline (DβH; Dopamine-β-hydroxylase, a marker for noradrenergic neurones), GABA (GAD; Glutamic acid decarboxylase, a marker for γ-amino butyric acid neurones), CRH, AVP, β-endorphin and GnRH.

Primary antisera	Dilution	Source	Reference
Anti-rabbit Fos	1:10,000	Oncogene Research Products, Cambridge, MA, USA	(Clarke *et al.*, 2001)
Anti-mouse ERα	1:50	Dako, Carpinteria, CA, USA	(Pompolo *et al.*, 2003a)
Anti-mouse DβH	1:500	Chemicon Europe Ltd, Hampshire, UK	(Pompolo *et al.*, 2003b)
Anti-rabbit DβH	1:500	Protos Biotech, New York, USA	(Clarke *et al.*, 1999)
Anti-mouse GAD (terminals only)	1:250	Developmental Studies Hybridoma Bank, Iowa city, IA, USA	(Pompolo *et al.*, 2003a)
Anti-rabbit CRH	1:500	Prof G. Tramu, INRA, Paris Cedex, France	(Chaillou *et al.*, 2002)
Anti-rabbit CRH	1:500	Bachem, St Helens, Merseyside, UK	
Anti-rabbit AVP	1:2000	Immunostar, Wisconsin, USA	(Caston-Balderrama *et al.*, 1999)
Anti-rabbit β-endorphin	1:1000	Bachem	
Anti-rabbit β-endorphin	1:1000	Prof P. Petrusz, University of North Carolina, NC, USA	(Finley *et al.*, 1981)
Anti-mouse GnRH	1:1000	Dr H. Urbanski, Oregon Regional Primate Research Center, OR, USA	(Pompolo *et al.*, 2003a)

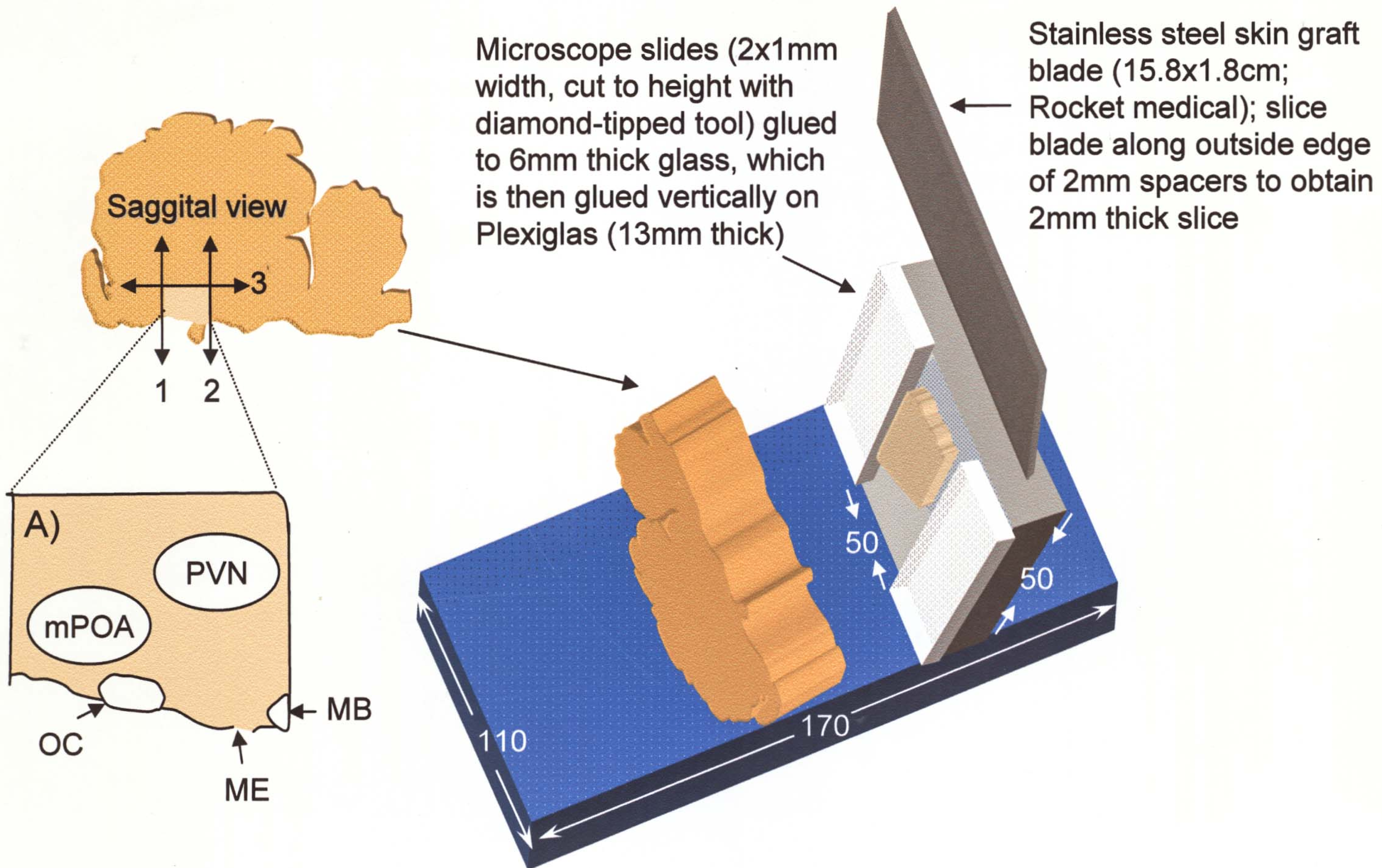


Fig 2.1 Schematic drawing of hypothalamic tissue slicer. Saggital view of ewe brain showing the position of first three cuts made with the skin graft blade and the final cut was made by placing medial aspect of the saggital half of brain against the block of glass. Measurements in mm. A) is the hypothalamic slice containing medial preoptic area (mPOA) and paraventricular nucleus (PVN). Abbreviations, MB, mammillary bodies; ME, median eminence; OC, optic chiasma.

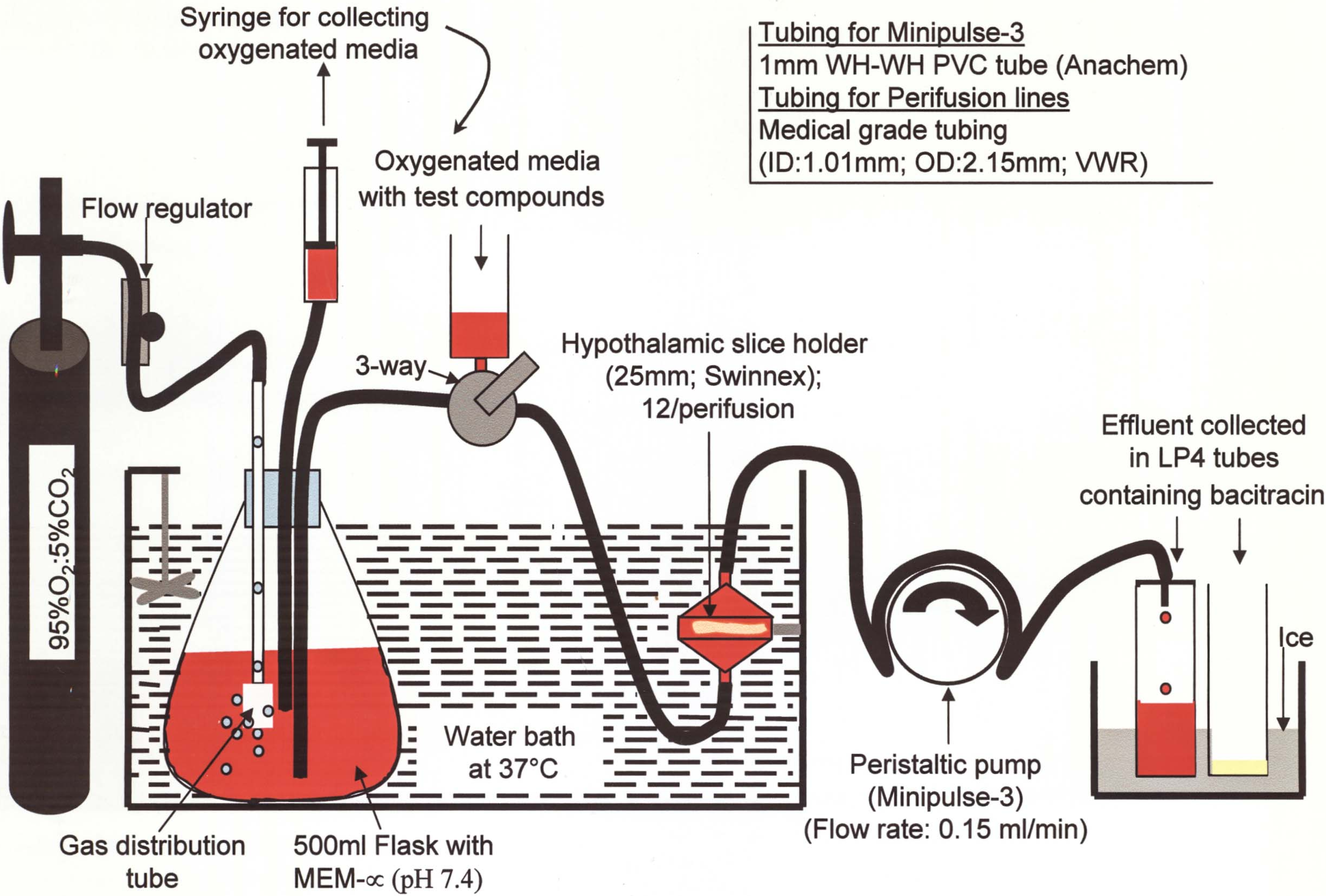


Fig. 2.2 Diagrammatic representation of the perifusion assembly for hypothalamic slices

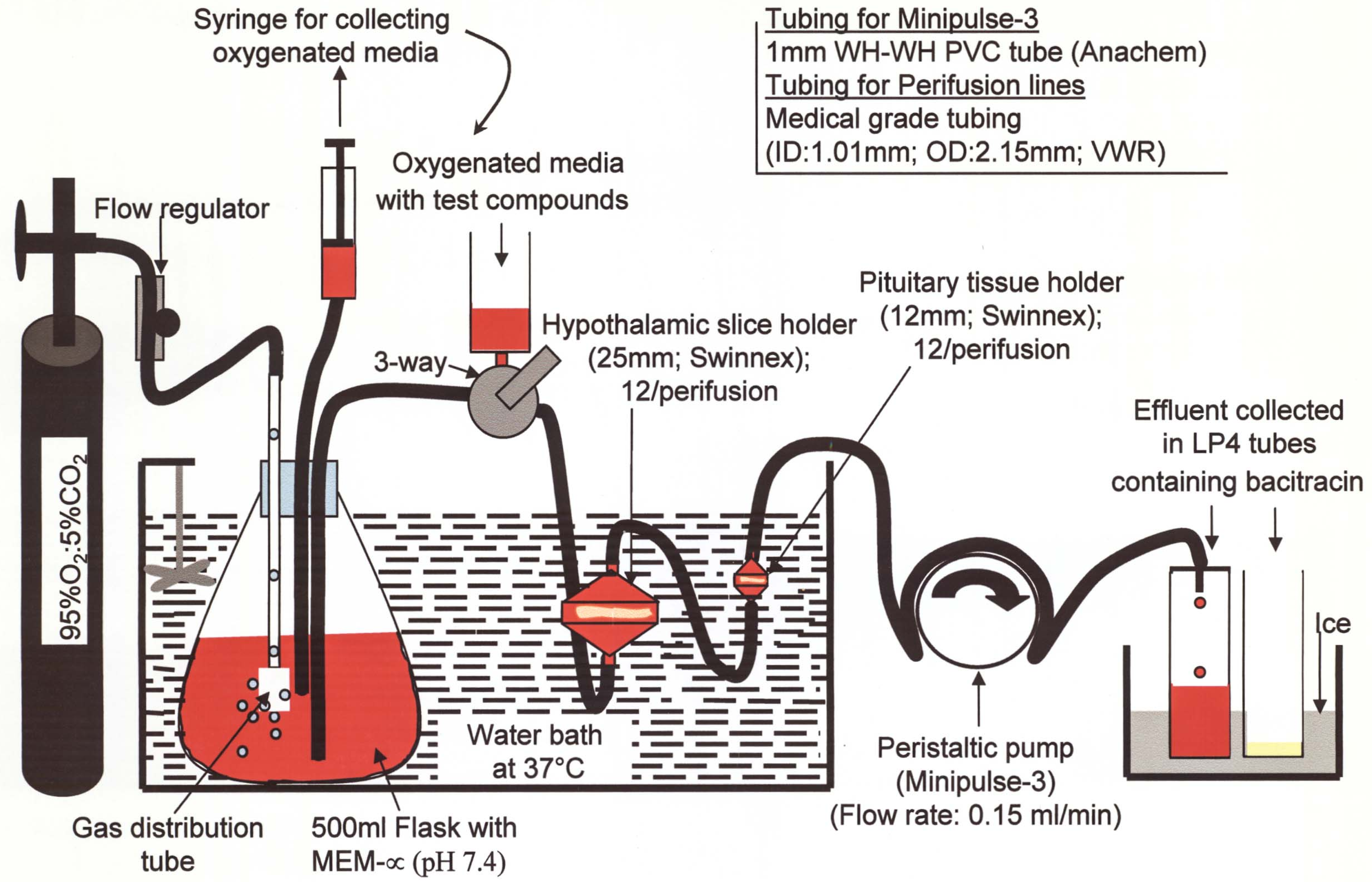


Fig. 2.3 Diagrammatic representation of the perifusion assembly for pituitary tissue in series with hypothalamic slices

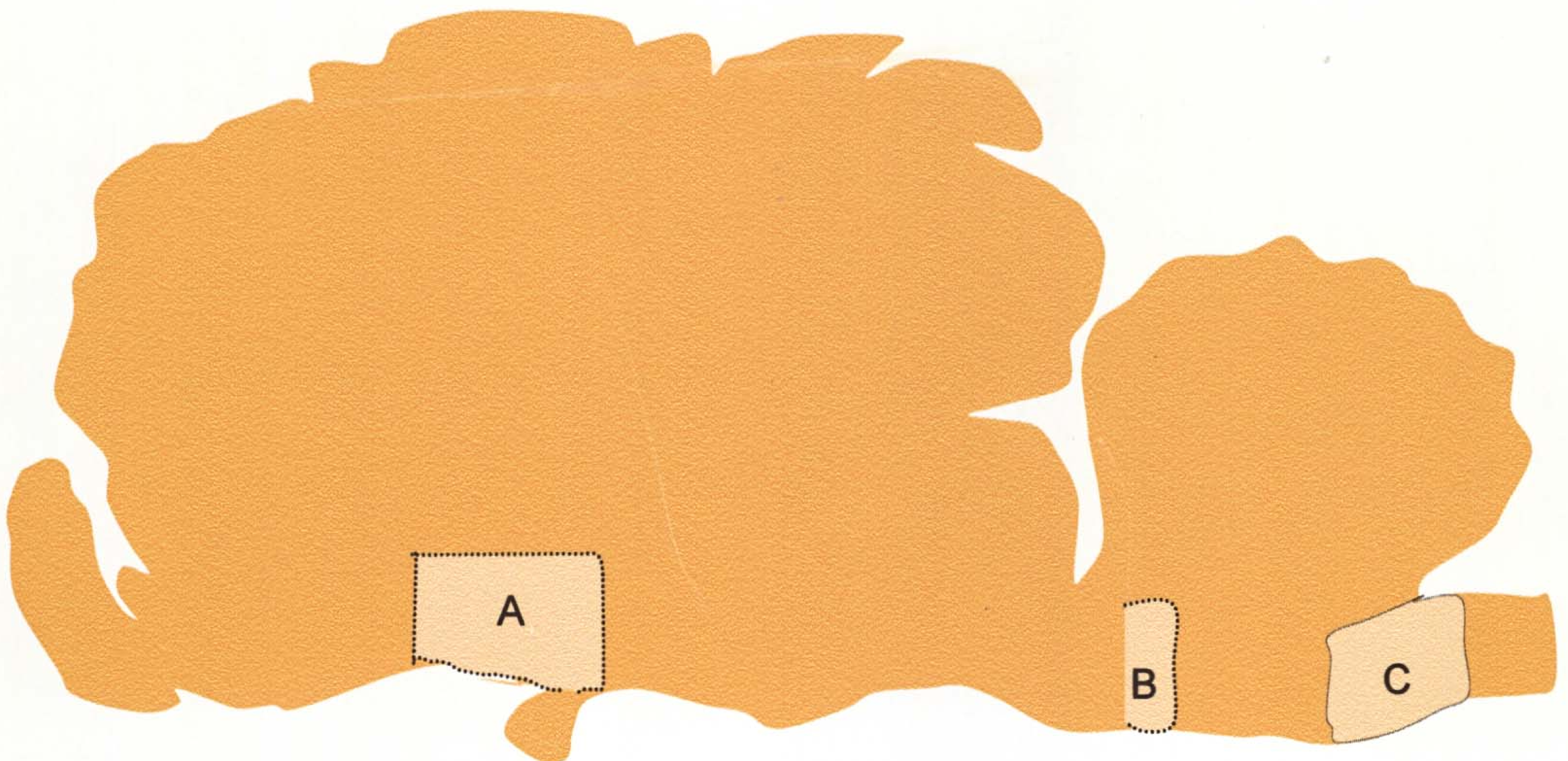


Fig 2.4 Schematic diagram of the ewe brain showing the area of A) hypothalamus, B) rostral and C) caudal brainstem used for immunohistochemistry studies.

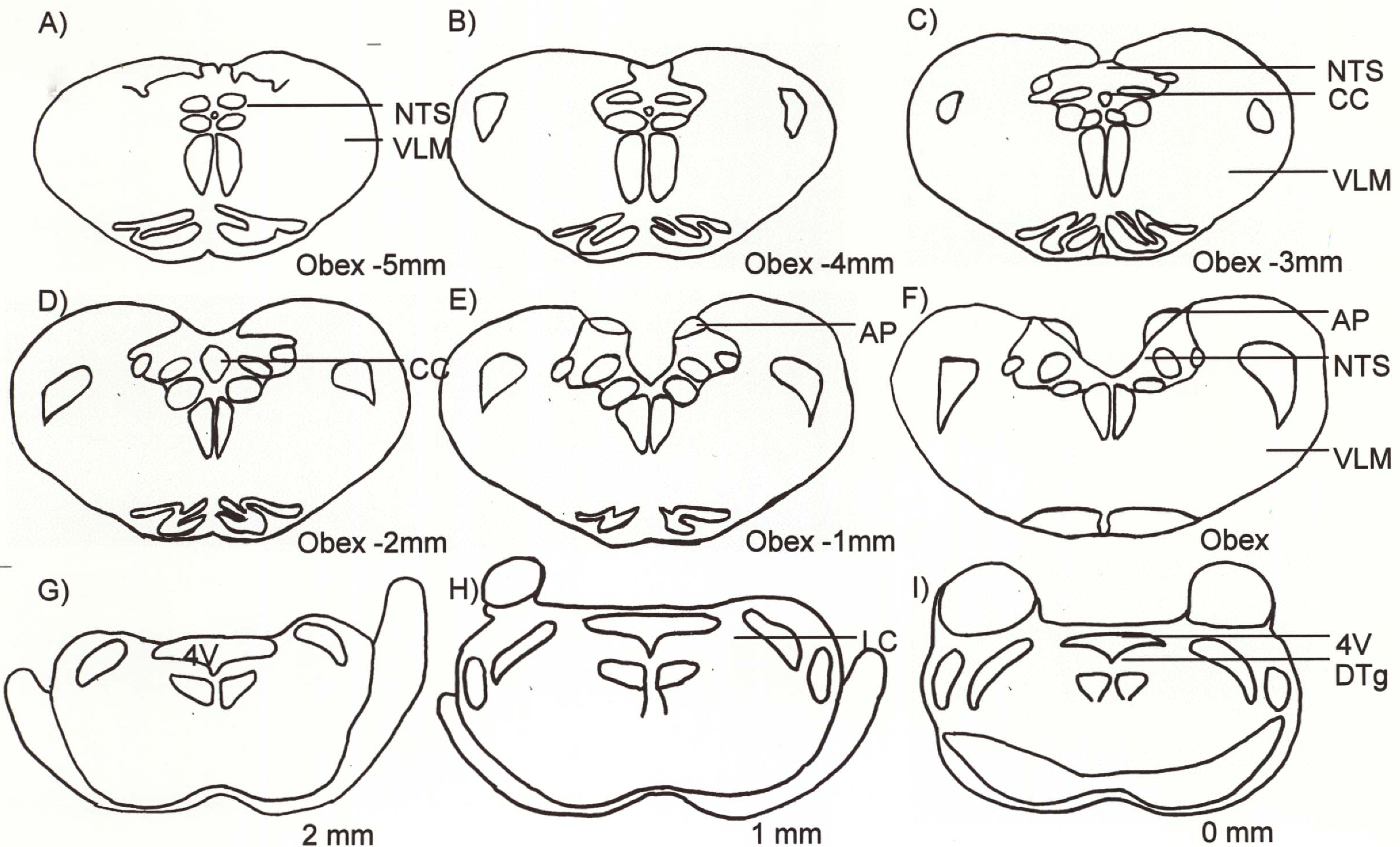


Fig 2.5 Schematic drawings of coronal sections (A-I, caudal to rostral) though the ewe brainstem. A-F) Caudal- and G-I) Rostral-brainstem, from caudal to rostral. Representative maps of the cells examined are shown in chapter 10. Abbreviations: AP, area postrema; cc, central canal; DTg, dorsal tegmental nucleus; LC, locus coeruleus; NTS, nucleus of solitary tract; VLM, ventrolateral medulla; 4V, fourth ventricle. Scale Bar, 5 mm

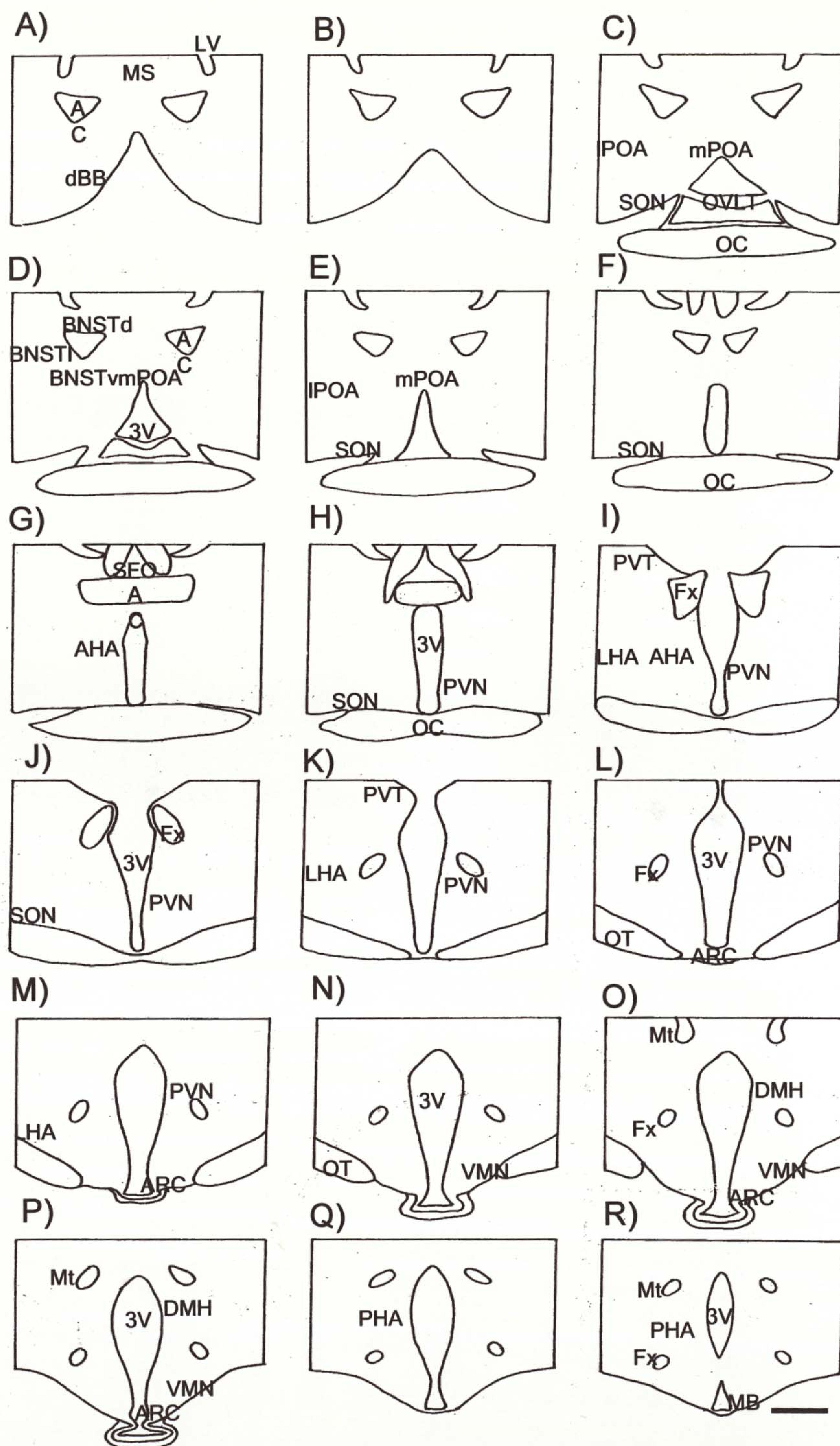


Fig 2.6 Schematic drawings of coronal sections through the ewe hypothalamus from rostral to caudal levels. Sections from A to N at 280μm and O to R at 960μm interval. Representative maps of the cells examined are shown in the chapters 9 and 10. Scale bar, 5mm. Abbreviations: AC, anterior commissure; AHA, anterior hypothalamic area; ARC, arcuate nucleus; BNST, bed nucleus of stria terminalis; dBB, diagonal band of broca; DMH, dorsomedial hypothalamus; Fx, fornix; LHA, lateral hypothalamic area; lPOA, lateral preoptic area; LV, lateral ventricle; MB, mammillary body; mPOA, medial preoptic area; MS, medial septum; MT, mammillothalamic tract; OC, optic chiasma; OT, optic tract; OVLT, organum vasculosum of lamina terminalis; PHA, posterior hypothalamic area; PVN, paraventricular nucleus of hypothalamus; PVT, paraventricular nucleus of thalamus; SFO, subfornical organ; SON, supraoptic nucleus; VMN, ventromedial nucleus; 3V, third ventricle.

Chapter 3

Oestradiol stimulates the release of AVP and GnRH from ewe hypothalamus *in vitro*

Oestradiol stimulates the release of AVP and GnRH from ewe hypothalamus *in vitro*

Abstract

Oestradiol sensitises reproductive axes to stress *in vivo*. Our current aim is to investigate whether E_2 influences hypothalamic AVP and GnRH release *in vitro*. Ten minutes after ewe sacrifice, saggital midline hypothalamic slices (from the anterior preoptic area to mediobasal hypothalamus with the median eminence, 2 mm thick, 2 per ewe) were dissected, placed in oxygenated MEM-α at 4°C and within 2 h were singly perifused at 37°C with oxygenated MEM-α (pH 7.4; flow rate 0.15 ml/min) alone (vehicle; n=15 perifusion chambers), with low (6 pg/ml; n=14) or high E_2 (24 pg/ml; n=13). After 5 h equilibration, 10 min fractions were collected for 3 h with exposure to 100 mM KCl for 10 min within the last hour. Concentrations of AVP and GnRH were measured by RIA. Baselines for AVP and GnRH were 7.0±1.1 and 17.4±0.8 pg/ml, respectively. Basal values with low E_2 were similar to vehicle for AVP (7.5±1.2 pg/ml) and GnRH (17.5±1.1 pg/ml). However, high E_2 increased basal AVP (11.7±1.4 pg/ml; $p<0.05$) and GnRH (23.7±1.4 pg/ml; $p<0.05$). After KCl, AVP and GnRH respectively, increased ($p<0.05$) to 25.6±7.5 and 38.2±5.6 (vehicle), 26.3±7.5 and 23.6±2.1 (low E_2) and 24.1±5.4 and 41.3±6.6 pg/ml (high E_2). After KCl, maximum values of AVP occurred at 20 and GnRH at 30 min. Conclusion: the *in vivo* effect of E_2 may be caused by a central action on the hypothalamus to augment AVP and GnRH release.

Introduction

There is considerable evidence to show that stress reduces gonadotrophin releasing hormone (GnRH), luteinising hormone (LH) and oestradiol (E_2) in ewe and many other species (Rivier & Rivest, 1991; Smith & Dobson, 2002). Conversely, circulating E_2 also regulates a wide variety of CNS functions, including stress responses via the hypothalamic-pituitary-adrenal (HPA) axis, and neuroendocrine

control of the reproductive hypothalamic-pituitary-gonadal (HPG) axis (Handa *et al.*, 1994; Herbison, 1998). These effects of E_2 are hypothesised to take place directly within the hypothalamus upon certain neuronal populations. Arginine vasopressin (AVP) neurones are important within the stress axis (Smith *et al.*, 2003a) whereas the focal point of E_2 feedback on the reproductive axis remains the GnRH neuronal population (Herbison, 1998). Precisely how E_2 exerts these effects on the hypothalamus remains unresolved.

Most studies *in vivo* suggest that E_2 increases circulating AVP concentrations (Skowsky *et al.*, 1979; Yamaguchi *et al.*, 1979; Peysner & Forsling, 1990). The recently identified oestradiol receptor (ER)-β colocalises with AVP neurones both in the magnocellular and parvocellular subdivisions of the paraventricular nucleus (PVN) (Isgor *et al.*, 2003b). However, E_2 has no effect on AVP mRNA through ERβ activation (Shapiro *et al.*, 2000) and, moreover, a pure E_2 antagonist microinjected into the PVN suggests that the dominant ERβ in the PVN suppress HPA responses to stress (Isgor *et al.*, 2003a). Therefore, an emerging possibility is that rapid non-genomic actions of E_2 may promote release of AVP by direct actions within the hypothalamus (Wang *et al.*, 1995).

Numerous studies have elucidated the biphasic control of GnRH neurones by E_2 (Levine, 1997; Herbison, 1998). During the late luteal and early follicular phases, low E_2 concentrations exert a negative feedback effect on GnRH secretion. In contrast, during the late follicular phase, rising E_2 concentrations increase GnRH release (Goodman, 1994). Oestradiol may influence GnRH release through ER (α or β) located on GnRH neurones or through modulation of afferent neurotransmitter inputs such as γ-amino butyric acid (GABA). The balance of evidence favours the neurotransmitter option (Petersen *et al.*, 2003).

To extend our previous *in vivo* observations in ewe (Smith *et al.*, 2003a), our objective was to establish an *in vitro* system to examine in greater detail the mechanisms by which E_2 regulates hypothalamic AVP and GnRH release. For this purpose, we have developed an *in vitro* perifusion system to examine the release of AVP and GnRH in response to low or high concentrations of E_2 applied directly to isolated hypothalamic tissue.

Materials and methods

In vitro perifusion

Hypothalamic slices were collected and perifused based on system described previously (chapter 2). Following a five hour equilibration period to attain basal AVP and GnRH release, 10 min serial effluent fractions (1.5 ml) were collected for next three hours. Samples were divided into separate volumes and immediately prepared for AVP assay and GnRH extraction.

Treatments

Hypothalamic slices were perifused with medium containing low (6 pg/ml) or high (24 pg/ml) concentrations of E_2 (Phogat *et al.*, 1997). Medium perifused without E_2 but containing appropriate ethanol concentrations served as a vehicle control.

Hypothalamic slice viability

Slice viability was verified as described previously (chapter 2).

GnRH assay

GnRH was measured by radioimmunoassay (chapter 2). Non-specific binding was less than 2.3±0.3%, sensitivity of the assay averaged 5.7 pg/ml. Inter- and intra-assay coefficients of variation were 8.5% and 6.0%, respectively.

AVP assay

AVP was measured by radioimmunoassay (chapter 2). Nonspecific binding was less than 2.2±0.1%, sensitivity of the assay averaged 3.7 pg/ml. Inter- and intra-assay coefficients of variation were 9.2% and 6.6%, respectively.

Statistical analysis

Basal release was defined as the mean of AVP and GnRH release in effluent fractions collected for 110 min during media perifusion without, or with low or high, E_2 prior to KCl challenge. Data were transformed to $\log_{10}$ before statistical analysis to

achieve homogeneity of variance among treatments. Differences between treatment groups were analyzed by generalized linear model (GLM ANOVA) procedures and Tukey's pairwise comparison. Interactions between sheep, ovarian status, left or right hemi-hypothalamic slice and perifusion chamber location were also analyzed by GLM ANOVA. Student's paired *t* tests (two tail) were used to examine differences between individual means of the pre- (120 min) and post- (130-180 min) KCl periods. Probabilities of less than 0.05 were considered significant and only these differences were reported. Data were expressed as means ± SEM. Statistical analyses were performed using Minitab release 13.20 statistical software (Minitab Inc., State College, PA, USA).

Results

Overall AVP and GnRH release during the first 110 min (baseline) was higher after perifusion with medium containing high (24 pg/ml) E_2 than after perifusion with low (6 pg/ml) E_2 or vehicle (no E_2; Figs 3.1 and 3.2). There were no interactions between sheep, ovarian status, left/right hemi-hypothalamic slice or perifusion chamber location on release of AVP or GnRH; i.e. all slices from all animals and perifusion chamber locations responded similarly. Baselines for AVP in the vehicle (7.0±1.1 pg/ml; n=15) and low E_2 (7.5±1.2 pg/ml; n=14) group were similar. However, high E_2 increased basal AVP (11.7±1.4 pg/ml; n=13) release. Similarly, baselines for GnRH in the vehicle (17.4±0.8 pg/ml; n=11) and low E_2 (17.5±1.1 pg/ml; n=11) group were similar. But, high E_2 increased basal GnRH (23.7±1.4 pg/ml; n=12) release.

The depolarizing stimulus (100 mM KCl) at 120 min increased AVP release similarly in all groups. Exposure to KCl increased AVP release to maximum values of 25.6±7.5, 26.3±7.5 and 24.1±5.4 pg/ml in the vehicle, low or high E_2 groups, respectively at 140 min. Similarly, GnRH release following KCl exposure increased to maximum values of 38.2±5.6, 23.6±2.1 and 41.3±6.6 pg/ml for vehicle, low or high E_2 groups, respectively. However, the GnRH response to KCl in the low E_2 group was

smaller compared to the vehicle and high E_2 groups. Interestingly, the maximum response of GnRH at 150 min was delayed compared to the AVP response at 140 min.

Low power light micrographs of ewe hypothalamic slices after perifusion are shown in Fig 3.3. Neuronal cell bodies were divided into three classes: Class I - tightly packed cells characterized by light, well-formed nuclei, surrounded by a thin shell of cytoplasm comprising the cell body; Class II cells were fairly dark but relatively healthy looking; Class III cells were vacuolated with large extracellular spaces. Cells from the ventricular surface were mainly Class I and distinctly different from the Class II and III cells from the middle of the 2 mm thick slice.

Discussion

To our knowledge, the present study is the first demonstration that E_2 stimulates AVP and GnRH release from ewe hypothalamic slices *in vitro*. These results are in agreement with *in vivo* studies in sheep reporting an increase in hypothalamic AVP concentrations after E_2 treatment (Wood *et al.*, 2001), and greater release of GnRH into hypophyseal portal blood under the influence of high E_2 concentrations (Moenter *et al.*, 1990).

In vitro perifusion was the system of choice to determine the direct effects of E_2 on specific areas of the hypothalamus because of the complex effects of E_2 in the whole animal (brainstem, anterior and posterior pituitary, and haemodynamics). In the present *in vitro* perifusion system, more AVP was released than GnRH in the 5 h equilibration. An increased initial efflux during the equilibration period (not shown) may have been due to increasing the temperature from 4°C during collection to 37°C in the perifusion chambers (Ramirez *et al.*, 1980) or due to leakage of peptides from the cut fibres of the hypothalamo-hypophyseal pathway passing through internal zone of the median eminence. However, the greater basal release of GnRH than AVP was maintained throughout the basal period. This is surprising due to the low number of GnRH cells scattered from the preoptic area to mediobasal hypothalamus compared to many more AVP cells in the PVN.

Evidence that the slices were healthy and functional is provided by the KCl challenge and histological evaluation. Depolarization with KCl is a highly potent physiologically relevant membrane-mediated stimulus for hormone release because it triggers sodium and calcium influx, thereby leading to release of hormones (Hartter & Ramirez, 1980). Furthermore, depolarization of neurosecretory terminals is a preliminary event in neuropeptide release (Ludwig *et al.*, 2002). Therefore, the ability of slices to respond to depolarization was a valuable indicator of slice viability. Hypothalamic slices maintained in our perifusion system were viable for at least 10 h after collection because AVP and GnRH release increased when slices were exposed to depolarization. The cause of the delayed response of GnRH to KCl compared to AVP in the present study is not fully understood but provides a further evidence for a differential active response by living neurones in contrast to simultaneous release that could have been expected if the dead cells simply lysed.

Electrophysiological studies suggest that the firing rate of GnRH neurones is markedly lower than PVN magnocellular neurones (Nunemaker *et al.*, 2002). Furthermore, GnRH neurones are not organized in discrete nuclei within the hypothalamus but are rather diffusely distributed (Lehman *et al.*, 1986) and may not be readily accessible unlike AVP neurones concentrated in the PVN near to the surface of the slices. Therefore, potassium may take longer to reach GnRH neuronal cell bodies and after diffusing into tissue in media may be diluted to a lower molar concentration at the GnRH perikarya. In addition, a change in spontaneous neuronal activity may be responsible because neuronal firing is a function of tissue slice thickness. The percentage of neuronal cells with low firing increases with the distance from the slice surface; however, neurones exhibit robust firing activity near the slice surface (Burgoon *et al.*, 1997).

Histological evaluation of slices was also used as a reliable indicator of slice health. The presence of Class I cells on the ventricular surface indicated that the slices were morphologically healthy and were maintained up to the end of perifusion, being able to carry out fairly complex physiological processes. However, the presence of degenerated cells near the centre of slices was probably the result of hypoxia and ischemia due to media diffusion distance limitations. Hypothalamic slices, in the

absence of an intact vascular system, rely on passive media perfusion from the slice edges towards the centre. Some deterioration near the center of slice was also revealed by the increased amount of extracellular space between cells and degenerative changes in the cell cytoplasm. Nevertheless, the slices maintained functional integrity as displayed by the differential responses to KCl.

The stimulatory action of high E_2 concentrations on AVP release from hypothalamic slices *in vitro* complements other *in vivo* studies in various species. A stimulatory effect of E_2 on plasma AVP has been described and conversely ovariectomy decreased circulating concentrations of this peptide in rats (Skowsky *et al.*, 1979; Yamaguchi *et al.*, 1979; Peysner & Forsling, 1990). At the level of the hypothalamus, an increase in AVP content occurred in the PVN and SON after an injection of E_2 to ovariectomized rats (Hatton *et al.*, 1992; Patchev *et al.*, 1995). Oestradiol treatment also augmented hypothalamic tissue AVP concentrations in fetal and adult sheep (Wood *et al.*, 2001).

In vitro studies in rats have demonstrated that E_2 stimulates hypothalamic AVP release (Forsling, 1993). The value of the present results in the ewe lies in our interest to support our *in vivo* observations to elucidate the mechanisms by which E_2 sensitizes the reproductive neuroendocrine axis to the inhibitory influence of stress. Stress stimulates AVP release in the rat and ewe (Muret *et al.*, 1992; Smith *et al.*, 2003a) but has suppressive effects on LH release only in presence of E_2 in the rat, unlike the sheep, thus negating direct extrapolation between rat and ewe data (Cates *et al.*, 1999; Dobson *et al.*, 1999). AVP responsible for ACTH secretion is produced from the parvocellular cell bodies in the PVN and is secreted into pituitary portal capillaries from axon terminals in the external zone of median eminence (Antoni, 1993). The methodology used in the present perifusion experiments does not distinguish the cellular origin of the AVP, whether magnocellular or parvocellular. However, *in vivo* studies also show that magnocellular neurones are capable of releasing AVP from axons at the level of the median eminence which gains access to portal blood and contributes to regulation of pituitary ACTH secretion (Holmes *et al.*, 1986; Buma & Nieuwenhuys, 1987; Irvine *et al.*, 1989; Antoni *et al.*, 1990; Dohanics *et al.*, 1991; Tannahill *et al.*, 1991; Wotjak *et al.*, 1996). In addition, ACTH secretion after osmotic

stimulation of the SON provides evidence for a direct involvement of magnocellular AVP neurones in the regulation of HPA axis activity (Wotjak *et al.*, 2002). Therefore, the present study is very important because it provides evidence that, in the ewe, E_2 can augment the activity of the HPA axis through AVP release from magnocellular and/or parvocellular cells and their axons within the hypothalamus rather than acting on magnocellular AVP terminals in the adenohypophysis.

The potential effects of E_2 on the secretary activity of AVP neurones through ERs are controversial, mainly due to differing interpretations based on the location of receptors *versus* evidence for their activity. ERs are differentially expressed among AVP neurones in different brain regions in the rat. AVP neurones in the SON and PVN express only ERβ, while AVP neurones expressing both ERs (α and β) are in the BNST, medial amygdala and periventricular preoptic nucleus (Shughrue *et al.*, 1997a; Simonian & Herbison, 1997; Alves *et al.*, 1998; Laflamme *et al.*, 1998; Isgor *et al.*, 2003b). As far as activity is concerned, ERα respond to E_2 by increasing AVP mRNA transcription in the BNST and the amygdala; while in SON and PVN neurones, that express ERβ, the opposite action of E_2 results suppression of AVP mRNA (Shapiro *et al.*, 2000; Nomura *et al.*, 2003). Also, *in vitro* studies suggest that E_2 has inhibitory action on AVP release through ERβ (Somponpun & Sladek, 2002). In ewe, a few neurones (unknown phenotype) lightly immunopositive for ERα are found in the PVN (Lehman *et al.*, 1993). With all this evidence, it is very unlikely that the increased AVP release observed in the present study could occur via nuclear receptor action because stimulatory ERα are not present on AVP neurones and ERβ are inhibitory to AVP release. The rapid effects of E_2 observed in the present study suggest that E_2 induced changes in AVP release by non-genomic mechanisms but these are not currently well understood in parvocellular or magnocellular neurones. High doses of E_2 induce rapid exocytosis of AVP from dendrites and cell bodies of magnocellular neurones in rat SON *in vitro* (Wang *et al.*, 1995). A similar mechanism may operate for the parvocellular AVP neurones. Oestradiol could exert these rapid non-genomic membrane effects by modulating ion channels, activating G-protein-linked receptors or other cellular components such as second messenger systems (Falkenstein *et al.*, 2000).

In present study, the basal secretion of GnRH was stimulated by high E_2 concentrations, with low E_2 concentrations having no apparent effect. It is well established that E_2 at high plasma concentrations prior to the preovulatory surge, stimulates the release of GnRH into hypophyseal portal blood (Moenter *et al.*, 1990). The mechanisms of alterations in the excitability of GnRH neurones under the influence of E_2 are yet to be fully elucidated. Historically GnRH neurones have been thought not to contain the classical ERs (Lehman & Karsch, 1993) and, therefore, it is unlikely that E_2 may directly influence the GnRH neural system. However, several recent reports indicate that GnRH neurones express the transcript and protein for both α- and β-isoforms of the ER (Butler *et al.*, 1999; Skynner *et al.*, 1999; Kallo *et al.*, 2001). But there is still a question whether activation of these receptors in GnRH neurones alters the functioning of these cells. In contrast, the role of E_2 sensitive inhibitory GABA interneurones providing synaptic input to $GABA_A$ receptor colocalising GnRH neurones appears more convincing (Leranth *et al.*, 1985; Herbison *et al.*, 1993b; Sim *et al.*, 2000). High E_2 concentrations decrease GABA turnover which in turn facilitates activation of GnRH neurones leading to the generation of a GnRH/LH surge *in vivo* (Robinson *et al.*, 1991; Wagner *et al.*, 2001). Furthermore, E_2 facilitates the stimulatory action of noradrenaline on GnRH secretion by increasing the electrical activation of GnRH neurones and by altering adrenoreceptor coupling within the GnRH network (Herbison, 1997a). In the present study, high E_2 concentrations may have altered the balance between inhibitory and stimulatory inputs to the GnRH neurones to promote the basal release of GnRH. Furthermore, there was no evidence for any inhibition of low E_2 on GnRH release *in vivo* that usually occurs in combination with luteal progesterone (Goodman, 1994). Oestradiol suppresses GnRH secretion predominantly during the non-breeding season by removing permissive noradrenergic inputs and promoting inhibitory GABA inputs, however, the opposite is the case during breeding season (Clarke & Scott, 1993).

Finally, the dampened GnRH responses to potassium depolarization in the low E_2 group compared to robust firing of GnRH neurones in slices perifused with high E_2 could be due to E_2-induced modifications in the sensitivity of neuronal membrane to KCl. Gonadal steroid hormones alter electrical properties of neuronal membranes and

thus the firing of neurones (Joels, 1997). In support, peptide release is markedly greater after KCl stimulation of hypothalamic tissue taken from rats with high E_2 concentrations in oestrus stage (Arancibia *et al.*, 1997). Furthermore, hypothalamic tissue dissected from rats in diestrus-I stage with high concentrations of serum progesterone and E_2 is refractory to potassium challenge (Cabrera *et al.*, 1993). However, the high response to KCl challenge in vehicle control slices in comparison to low E_2 group remains unanswered.

In summary, the present results provide the first successful *in vitro* perifusion system for ewe hypothalamic slices. I*n vitro* stimulation of AVP and GnRH by E_2 may provide a useful system to address the E_2-dependent neuronal mechanism(s) controlling stress and reproductive activity.

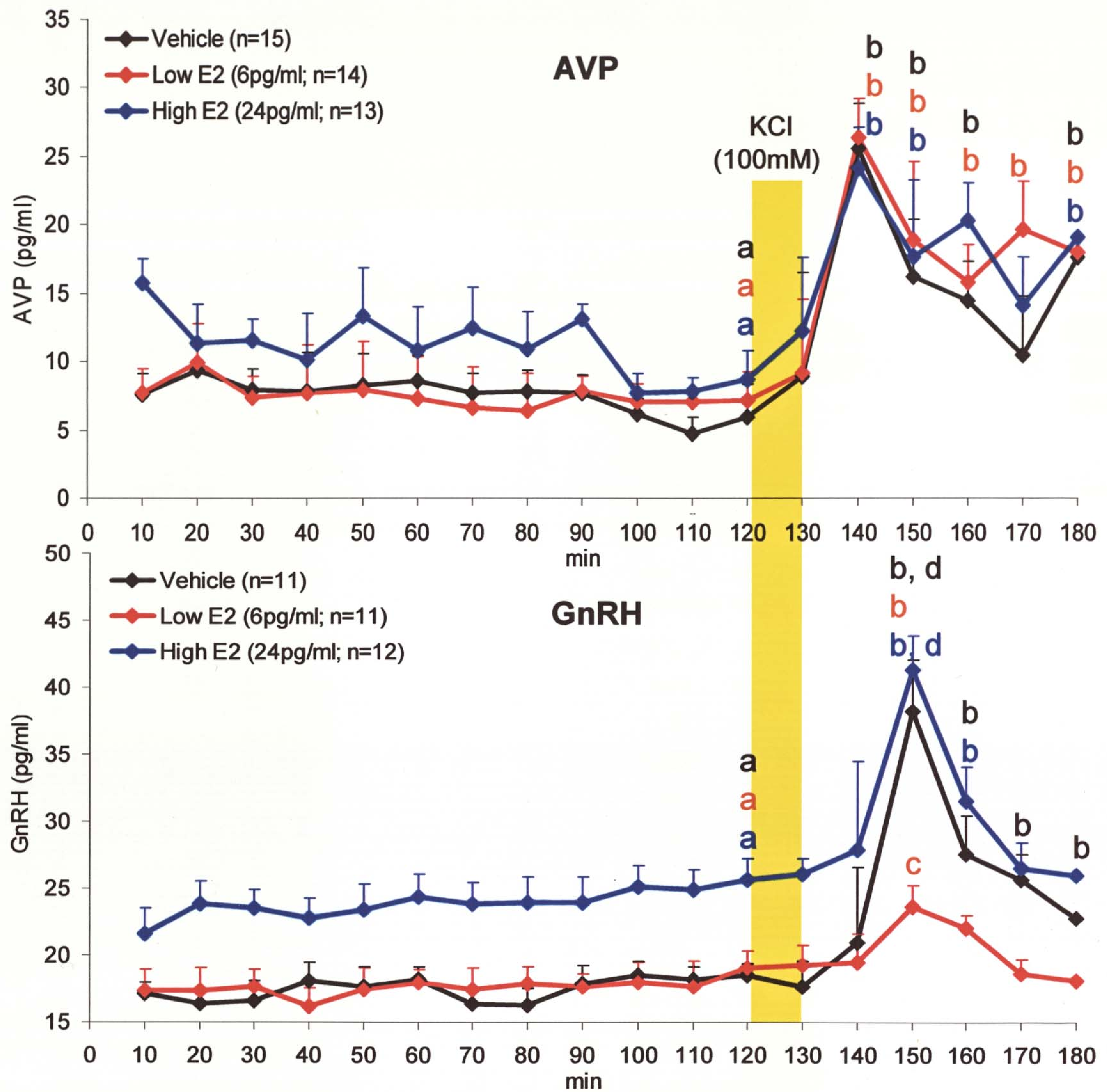


Fig 3.1 Mean concentrations (±SEM) of AVP and GnRH from ewe hypothalamic slices perifused with medium alone (vehicle) or containing low or high concentrations of oestradiol (E_2). Basal (0-110min) AVP and GnRH release was greater with high E_2 compared to vehicle or low E_2 exposure ($p<0.05$, GLM ANOVA). Maximum values of AVP and GnRH occurred 20 and 30min after KCl, respectively. $p<0.05$; within group (a *vs* b; Paired t-test); between groups (c *vs* d; Tukey's pairwise comparison).

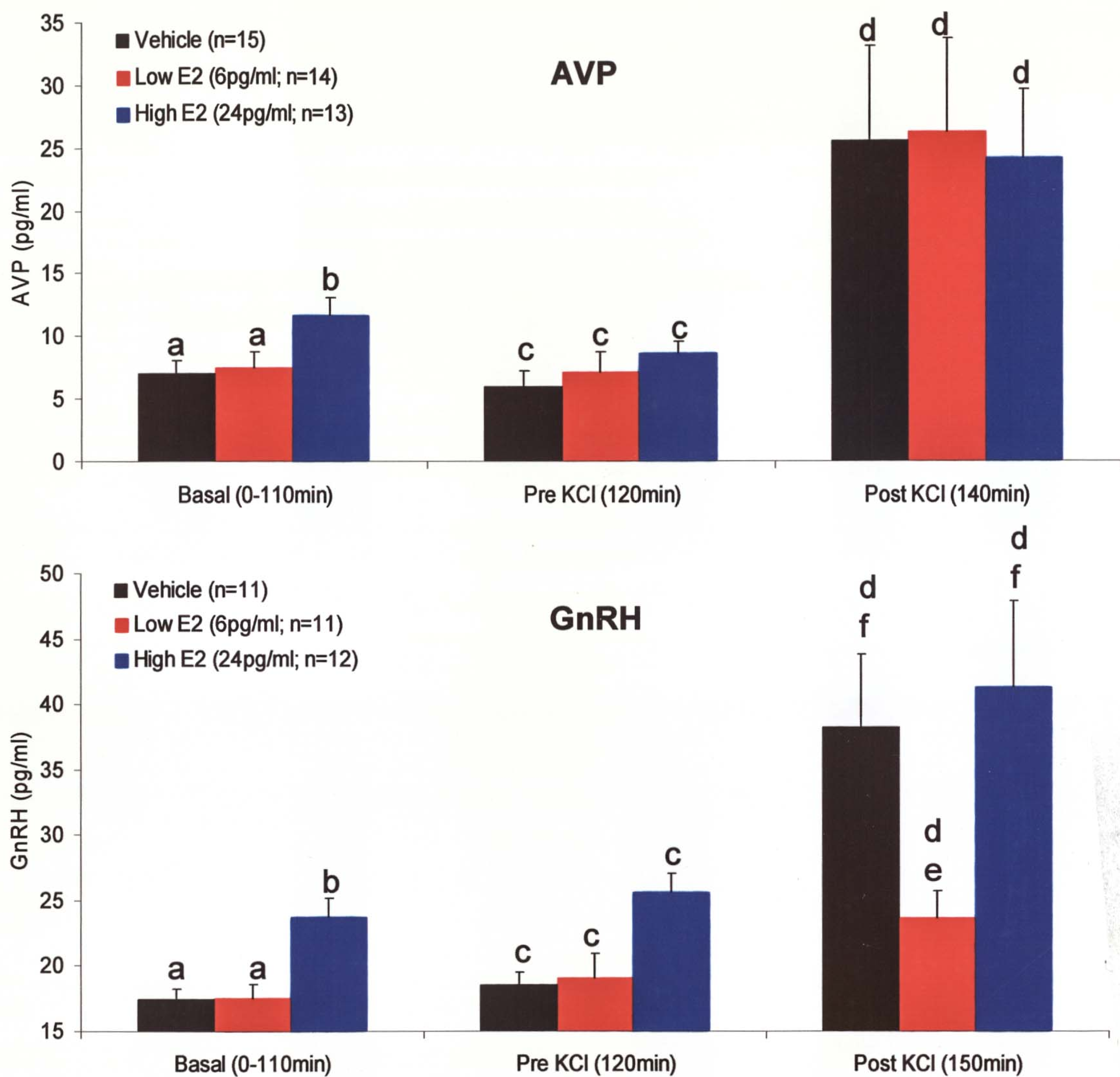


Fig 3.2 Mean concentrations (±SEM) of AVP and GnRH from ewe hypothalamic slices perifused with medium alone (vehicle) or containing low or high concentrations of oestradiol (E_2). Basal (0-110min) AVP and GnRH release was greater with high E_2 compared to vehicle or low E_2 exposure (a *vs* b, $p<0.05$, GLM ANOVA). Maximum values of AVP and GnRH occurred 20 and 30 min after KCl, respectively. $p<0.05$; within group (c *vs* d; Paired t-test); between groups (e *vs* f; Tukey's pairwise comparison).

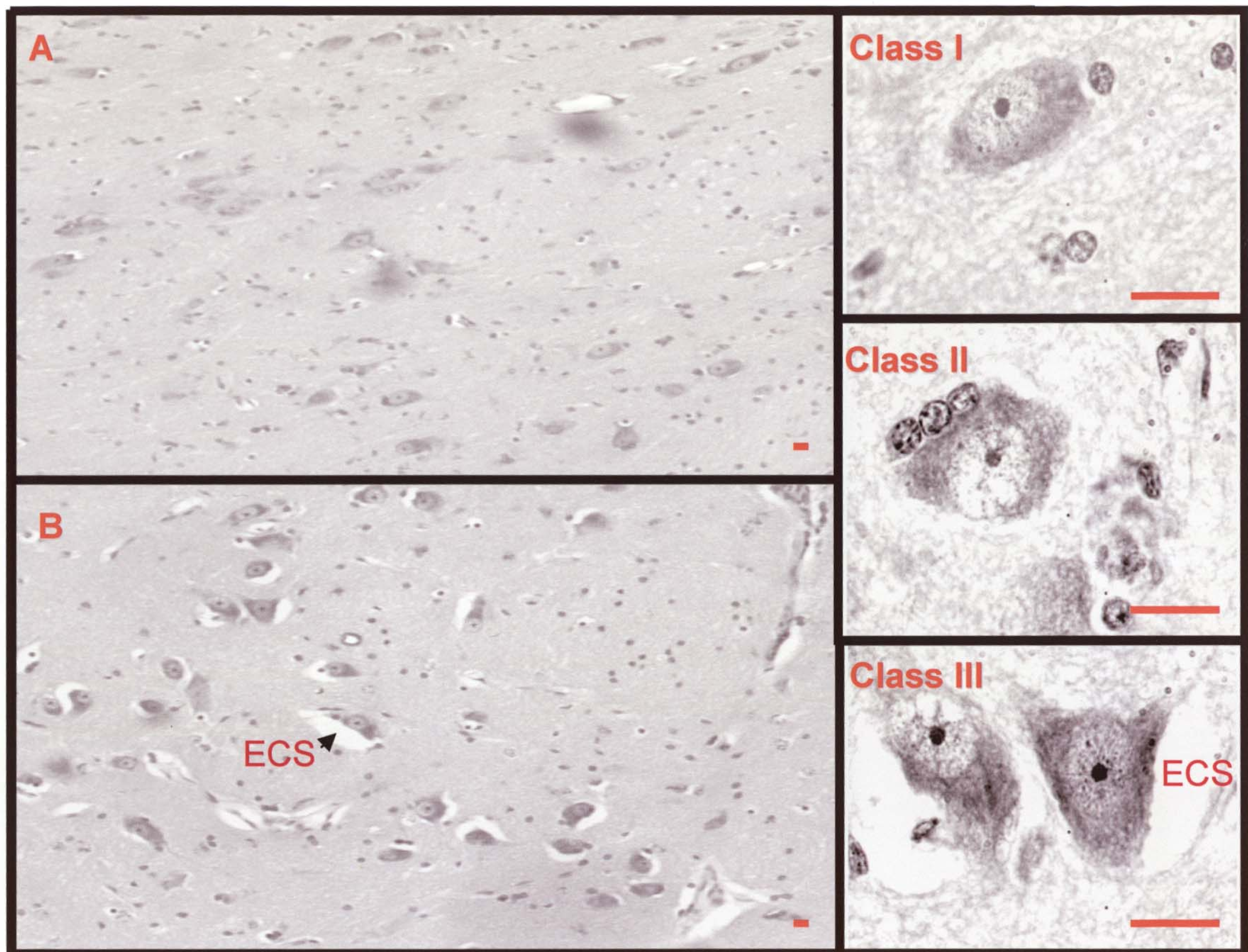


Fig 3.3 Low power light micrographs (10x) of ewe hypothalamic slices after perifusion from the ventricular surface (A) and middle of the slice (B). High power (100x) of the Class I, II, and III cells are also shown. Note the larger apparent extracellular spaces (ECS) in (B) with more Class II and III cells compared to (A) with Class I cells. Bar 20μm

Chapter 4

GABA control of arginine vasopressin release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

GABA control of arginine vasopressin release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

ABSTRACT

The present study aims to ascertain the influence of $GABA_{A\ or\ B}$ receptors on AVP release *in vitro* and determine whether E_2 modulates GABA-AVP interaction. Ten minutes after ewe sacrifice, saggital midline hypothalamic slices (from the anterior preoptic area to the mediobasal hypothalamus with the median eminence, 2 mm thick, 2 per ewe) were dissected, placed in oxygenated MEM-α at 4ºC and within 2 h were singly perifused at 37ºC with oxygenated MEM-α (pH 7.4; flow rate 0.15 ml/min), either with or without E_2 (24 pg/ml). After 4 h equilibration, 10 min fractions were collected for 4 h interposed with a 10 min exposure at 60 min to a specific $GABA_{A\ or\ B}$ receptor agonist or antagonist at various doses (0.1-10 mM). $GABA_A$ (muscimol; No E_2, n=4 perifusion chambers, with E_2, n=10) or $GABA_B$ (baclofen; No E_2, n=5, with E_2, n=12) agonists (10 mM) did not influence AVP concentrations. However, AVP release increased ($p<0.05$) 20-30 min after exposure to 10 mM $GABA_{A\ or\ B}$ antagonists (bicuculline, No E_2, n=4: from 4.6±0.7 to 33.0±0.4, With E_2, n=17: from 11.9±1.4 to 32.8±6.0; CGP52432, With E_2, n=14: from 14.0±2.6 to 28.8±3.9 pg/ml). At the end of the collection period, hypothalamic slices responded to KCl (100 mM) with AVP efflux ($p<0.05$). $GABA_B$ but not $GABA_A$ antagonist-stimulated AVP release was enhanced in the presence of E_2. In summary, AVP release is under the inhibitory influence of GABA input with further potentiation by E_2 through $GABA_B$ receptors *in vitro*.

INTRODUCTION

The hypothalamic paraventricular nucleus (PVN) contains neurosecretory cells that integrate stimulatory and inhibitory inputs to modulate hypophyseal hormone release (Herman *et al.*, 2003). Arginine vasopressin (AVP) neurones play a major role during the stress response in ewe (Smith *et al.*, 2003a). Stress-related inputs converge

on AVP synthesizing neurones which activate the neuroendocrine stress network by releasing AVP into the hypophyseal portal system to stimulate adrenocorticotropin (ACTH)-mediated release of corticosteroids from the adrenal cortex (Smith *et al.*, 2003a). Parvocellular AVP neurones are the primary source of input to the anterior pituitary, although studies in the rat suggest that paraventricular and supraoptic magnocellular AVP neurones, as well as projecting directly to the posterior pituitary, are also capable of releasing AVP from axons in the median eminence. Thus, AVP enters the portal blood and contributes to ACTH secretion (Holmes *et al.*, 1986; Buma & Nieuwenhuys, 1987; Antoni *et al.*, 1990; Wotjak *et al.*, 2002).

The dominant inhibitory neurotransmitter in the hypothalamus is γ-amino butyric acid, GABA (Herman *et al.*, 2003). Localized areas of the dorsomedial, anterior hypothalamic, preoptic (POA), and bed nucleus of the stria terminalis (BNST) contain GABA-producing cell bodies that send direct projections to the parvocellular and magnocellular neurones within the PVN in rats (Boudaba *et al.*, 1996). Morphological evidence indicates that half the cells in the medial parvocellular subdivision of the PVN receive synaptic inputs that are GABA immunoreactive (Miklos & Kovacs, 2002). Information regarding the *in vivo* interaction between GABA and the hypothalamic-pituitary-adrenal (HPA) axis is not available in the ewe; however, various studies in rats implicate inhibitory GABA control directly upon the HPA axis. Infusion of GABA into the third ventricle prevents stress-induced increases in corticosterone release (Makara & Stark, 1974). The excitability of AVP neurones is suppressed by GABA (Nissen & Renaud, 1994). Also, there is direct evidence for GABA innervation of AVP neurones in the rat PVN as well as supraoptic nuclei (SON) (Theodosis *et al.*, 1986; Decavel *et al.*, 1989). However, clarification is required for the ewe as it was not possible to identify close contacts between GABA terminals and AVP cell bodies in the PVN (chapter 9).

The GABA neurones exert inhibitory actions predominantly through $GABA_A$ receptors within the stress-related circuitry of the rat though the role of $GABA_B$ receptors has yet to be extensively investigated. Infusion of a $GABA_A$ receptor agonist, directly into the PVN, inhibits stress-induced *c-fos* expression and corticosterone release (Helmreich *et al.*, 1998; Kovacs *et al.*, 1998). Furthermore, *in*

situ hybridisation and electrophysiological studies suggest the presence of $GABA_A$ and $_B$ receptors on AVP neurones both in the PVN and SON (Fenelon & Herbison, 1995; Fenelon *et al.*, 1995; Slugg *et al.*, 2003; Li & Stern, 2004).

Oestradiol (E_2) augments HPA suppression of the hypothalamic-pituitary-gonadal (HPG) axis, but the regulatory pathways of this modulation are unclear (Chen *et al.*, 1992; Lund *et al.*, 2004). In the rat and monkey, AVP suppresses pulsatile luteinising hormone (LH) and thus E_2 release from the ovarian follicle decreases (Chen *et al.*, 1992; Muret *et al.*, 1992) but, conversely, there is growing evidence that E_2 modulates AVP neuronal activity. Intracerebroventricular injection of high doses of AVP suppresses LH secretion only in E_2-replaced ovariectomized (OVX) rats, with no effect in the absence of E_2 (Cates *et al.*, 1999). A significant increase in AVP concentrations in the hypothalamus occurs after E_2 treatment in sheep (Wood *et al.*, 2001), and we have recently demonstrated that elevated doses of E_2 stimulate AVP release from ewe hypothalamic slices *in vitro* (chapter 3).

Despite the functional impact of GABA on AVP neurones, the connection between E_2-modulated stress responses and modification by GABA still requires detailed investigation. The GABA neurones may provide the interface between E_2 and AVP neurones to modulate HPA activity and, as AVP neurones lack substantial numbers of E_2 receptors (ER), these effects may be mediated by afferent GABA projections from the POA and its subdivision, the BNST. In the ewe, one third of the preoptic ERα-positive neurones are also glutamic acid decarboxylase positive (GAD, a rate limiting enzyme for GABA) (Herbison *et al.*, 1993b). Within the rat POA, E_2 increases GABA release as well as $GABA_A$ receptor expression (Herbison *et al.*, 1995; Herbison & Fenelon, 1995). This same area extends stress-activated GABA projections to the PVN and lesions here increase HPA stress responses in the rat, i.e., consistent with an inhibitory input to the PVN (Boudaba *et al.*, 1996; Viau & Meaney, 1996).

The GABA system also appears important in the ewe in that GABA receptors are involved in E_2-induced suppression of the GnRH system predominantly during the non-breeding season (Clarke & Scott, 1993). Similar GABA functions may mediate E_2-induced reinforcement of HPA suppression on the HPG during the breeding

season. Indeed, we have evidence in the ewe that the suppressive effects of stress on LH secretion are greater in the presence of E_2 (Dobson *et al.*, 1999). The present study is designed to investigate whether a GABA receptor agonist or antagonist alters AVP release from the ewe hypothalamus and whether this relationship is influenced by the prevailing E_2 milieu. An *in vitro* approach was essential to avoid any confounding effects of GABA receptor compounds or E_2 on other body systems.

MATERIALS AND METHODS

In vitro perifusion

The perifusion was performed as described elsewhere (chapter 2). After a 4 h equilibration period, medium effluent fractions were collected for 4 h.

Treatments including $GABA_A$ or $_B$ receptor agonist/antagonist dose response curves

Hypothalamic slices were perifused with media either with or without E_2 (24 pg/ml, Sigma-Aldrich). After 60 min effluent collection, each slice was treated for 10 min with vehicle (MEM-α, without E_2, n=11 perifusion chambers; with E_2, n=7), or increasing doses of $GABA_{A\ or\ B}$ receptor agonists (muscimol-$GABA_A$, Sigma-Aldrich; without E_2, 0.1, 1.0 or 10 mM, n=4 each; with E_2, 0.1 or 1.0 mM, n=4 each; 10 mM, n=11; baclofen-$GABA_B$, Sigma-Aldrich; without E_2, 0.1 or 1.0 mM, n=4 each; 10 mM, n=5; with E_2, 0.1 mM, n=4; 1 mM, n=5; 10 mM, n=15) or antagonists (bicuculline-$GABA_A$, Sigma-Aldrich; without E_2, 0.1 mM, n=4; 1 mM, n=6; 10 mM, n=4; with E_2, 0.1 mM, n=4; 1 mM, n=8; 10 mM, n=17; CGP52432-$GABA_B$, Tocris-Cookson, Ellisville, MO, USA; without E_2, 0.1 mM, n=4; 1 mM, n=5; 10 mM, n=4; with E_2, 0.1 or 1.0 mM, n=4 each; 10 mM, n=14) by turning a three way stopcock to apply agonist/antagonist treatments held in 2 ml syringes. At the end of the perifusion all slices were exposed to a KCl (100 mM) challenge for 10 min after 180 min of effluent collection.

AVP assay

AVP was measured by radioimmunoassay (chapter 2). Sensitivity of the assay was 3.8 pg/ml. Inter- and intra-assay coefficients of variation were 7.8% and 6.0%, respectively.

Statistical analysis

Basal release, with or without E_2, was defined as the mean AVP release in effluent fractions collected for 60 min, prior to start of $GABA_{A\ or\ B}$ agonist/antagonist treatment. For dose response studies (Fig 4.1A-D, 4.2A-D), pre- or post-treatment values were calculated by averaging the AVP concentrations of individual chambers at 60 and 70 min for pre-treatment, and at 80 and 90 min for post-treatment. Values for two time points after treatments were averaged as the mean time of maximum response was variable between chambers. Similarly, pre- and post-KCl (Fig 4.1E-H, 4.2E-H) concentrations of AVP were calculated by averaging the 180 and 190, or 200 and 210 min fractions, respectively.

Data were transformed to $\log_{10}$ before statistical analysis to achieve homogeneity of variances among treatments. Comparisons among dose responses, within or between treatments were made by generalized linear model ANOVA and *post-hoc* Tukey's method for pairwise comparisons. Differences within individual chambers during pre- *versus* post-treatment periods were examined by Student's paired *t* test (two tail). Probabilities of less than 0.05 were considered significant and only these differences were reported. Data were expressed as means ± SEM. Statistical analyses were performed using Minitab release 13.2 statistical software (Minitab Inc., State College, PA, USA).

RESULTS

The mean basal (10-60 min) AVP release by hypothalamic slices perifused with medium containing E_2 was higher ($p<0.05$, 13.9±0.1 pg/ml, n=60) than slices perifused with medium containing no E_2 (8.2±0.1 pg/ml, n=28).

Dose responses: Comparing pre- and post-treatment AVP concentrations, within each treatment, there was no effect of E_2 on agonist or antagonist responses (Figs 4.1 and 4.2). Only after 1 or 10 mM $GABA_A$ antagonist (bicuculline) exposure, was there greater AVP release compared to pre-treatment values (asterisks; Figs 4.1C and 4.1D). In slices exposed to the $GABA_B$ antagonist (CGP52432), only the 10 mM dose stimulated AVP release compared to pre-treatment values in the presence of E_2 (asterisk; Fig 4.2D).

After the antagonists with or without or E_2, the 1 and 10 mM bicuculline responses were similar but greater than after vehicle or 0.1 mM bicuculline (Figs 4.1C, 4.1D; a *versus* b). After CGP52432 group, the response to 10 mM was greater but only in slices perifused with E_2 (Fig 4.2D; a *versus* b).

The release of AVP in response to bicuculline (1 or 10 mM) was greater than muscimol (1 or 10 mM) both in the presence (Figs 4.1B, 4.1D) or absence of E_2 (Figs 4.1A, 4.1C). But, CGP52432 (10 mM) stimulated more AVP release than baclofen (10 mM) only in the presence of E_2 (Figs 4.2B, 4.2D; c *versus* d).

Figures 4.3 and 4.4 emphasize the release patterns of AVP from hypothalamic slices during the post-treatment period (80-170 min) following exposure to 10 mM $GABA_{A\text{ or }B}$ agonist or antagonist (Fig 4.3, muscimol or bicuculline; Fig 4.4, baclofen or CGP52432) with or without E_2. The maximum stimulatory response to bicuculline occurred both in the presence and absence of E_2 at 80 and/or 90 min. The response to CGP52432 was greater only in the presence of E_2 at 80 and 90 min (Fig 4.4B).

As illustrated in Figs 4.1E-H and 4.2E-H and in Figs 4.3 and 4.4, hypothalamic slices responded to KCl (100 mM) with AVP efflux in vehicle and GABA receptor compound-treated groups. The lack of statistical significance in 6 out of 32 groups was due to large inter-chamber variation (Figs 4.1E, 4.1F, 4.2E, 4.2F and 4.2H). The KCl-evoked release tended to be higher in the slices exposed to E_2 than without but this was significant only in 6 out of 32 groups (Figs 4.1G, 4.1H, 4.2E and 4.2F).

DISCUSSION

Release of AVP from the hypothalamic slices was augmented by E_2 confirming our previous *in vitro* data (chapter 3) and in agreement with *in vivo* studies in sheep (Wood *et al.*, 2001).

The present study is the first to describe an inhibitory influence of GABA on AVP release from ewe hypothalamic slices *in vitro* with potentiation by E_2 through $GABA_B$ receptors. The selective $GABA_{A\ or\ B}$ antagonists, bicuculline or CGP52432, enhance AVP release but not the selective $GABA_{A\ or\ B}$ agonists, muscimol or baclofen. The effects of $GABA_{A\ or\ B}$ antagonists were dose-dependent, with AVP release occurring after lower doses of $GABA_A$ than the $GABA_B$ antagonist. Similar results are revealed in other species using *in vitro* hypothalamic blocks or organ-culture; $GABA_A$ antagonists stimulate AVP release whilst GABA itself is ineffective (Sladek & Armstrong, 1987; Hillhouse & Milton, 1989). All these results indicate that there is an inhibitory action of GABA within the hypothalamus. Additional support for the role of GABA comes from electrophysiological studies that demonstrate tonic GABA inhibitory control of magnocellular and parvocellular neurones through $GABA_{A\ and\ B}$ receptors (Boudaba *et al.*, 1996; Han *et al.*, 2002; Slugg *et al.*, 2003; Li & Stern, 2004).

In the present perifusion model, it is uncertain whether GABA antagonists interact directly with GABA receptors on the AVP neurones or via GABA receptors in local areas with projections to the PVN. The $GABA_A$ antagonist, bicuculline, injected *in vivo* into the dorsomedial nucleus of the hypothalamus potently increases PVN activation in rats (DiMicco *et al.*, 1996). Inversely, infusion of $GABA_A$ agonists into the PVN inhibits stress-induced *c-fos* expression and corticosterone release (Helmreich *et al.*, 1998; Kovacs *et al.*, 1998). The parvocellular and magnocellular compartments of the PVN are under differential GABA control in rats. With intracerebral administration of a $GABA_A$ antagonist, *c-fos* and AVP heteronuclear RNA expression are preferentially activated within parvocellular regions whereas the effect is absent in the magnocellular compartment (Cole & Sawchenko, 2002). However, electrophysiological studies suggest that both magnocellular and

parvocellular elements of the PVN are under similar $GABA_A$ receptor suppression (Tasker & Dudek, 1993).

The present study provides evidence for E_2 modulation of the interaction between GABA receptors and AVP release. Oestradiol did not influence AVP release after the exposure of hypothalamic slices to the $GABA_{A\ or\ B}$ agonists or the $GABA_A$ antagonist, although there was a greater response after the $GABA_B$ antagonist in E_2 perifused slices (Fig 4.5). To our knowledge, the present study is the first to demonstrate $GABA_B$ receptor mediation of E_2 action on AVP release in any species. However, there are a few studies in rats to indicate interaction of this steroid with the general GABA system. *In vivo* exposure to E_2 increases $GABA_A$ receptor mRNA expression, and GABA reuptake within the POA (Herbison *et al.*, 1995; Herbison & Fenelon, 1995). Administration of E_2 to OVX rats also increases the number of GABA binding sites in several CNS structures (Perez *et al.*, 1986). Oestradiol increases GABA turnover in rat medial POA slices *in vitro* and interestingly this area also sends GABA projections to the PVN (Herbison *et al.*, 1989; Boudaba *et al.*, 1996). Nevertheless, our observations do suggest that E_2 potentiates the $GABA_B$ receptor-mediated inhibitory GABA influence on the AVP neurones. Although, this appears to be in contrast to our working hypothesis of E_2-induced potentiation of stress responses through activation of HPA axis, a note of caution in the interpretation of these data must be given. *In vivo* evidence strongly suggests that GABA-GABA interactions in the brain may result in net disinhibition of the effector neurone. During acute stress, GABA efferents from the amygdala have the potential to disinhibit PVN-projecting GABA neurones in the BNST or POA, and thereby increase CRH activation (Herman *et al.*, 2003). The amygdala connections were lost in our hypothalamic slice model and secondly, during *in vivo* conditions, this E_2-induced GABA potentiation might be linked to stronger disinhibition mechanisms resulting in net HPA axis activation.

The observed inhibitory action of GABA through $GABA_{A\ and\ B}$ receptors does not totally concur with confocal immunohistochemistry in the ewe where GABA terminals were not in close contact with AVP cell bodies in the PVN (chapter 9). However, GABA innervation of AVP cell bodies and presence of GABA receptors on AVP neurones in the PVN has been suggested in rats (Decavel *et al.*, 1989; Fenelon &

Herbison, 1995; Fenelon *et al.*, 1995; Slugg *et al.*, 2003). Confocal studies may have overlooked small subpopulations of AVP cell bodies in close contact with GABA neurones. Also, methodologies with greater resolution need to be developed to examine very close contact between GABA terminals and fine AVP dendritic processes and axon terminals. Nevertheless, as GABA terminals were in the proximity of AVP cell bodies, GABA might inhibit AVP neurones by diffusion.

The responses to KCl depolarization demonstrated the viability of the hypothalamic slices. The potassium-evoked release of AVP from slices perifused with media containing E_2 tended to be higher which could be due to E_2-induced modifications in the sensitivity of neuronal membrane to KCl. Gonadal steroid hormones alter electrical properties of neuronal membranes and thus the firing of neurones (Joels, 1997).

Clearly, GABA receptors are involved in the suppression of AVP release within the hypothalamus in ewes. $GABA_B$ receptors in contrast to $GABA_A$ receptors are influenced by the presence of E_2. Overall, the present *in vitro* data suggest an intricate E_2-GABA-AVP interaction that might be involved in neural responses to stress.

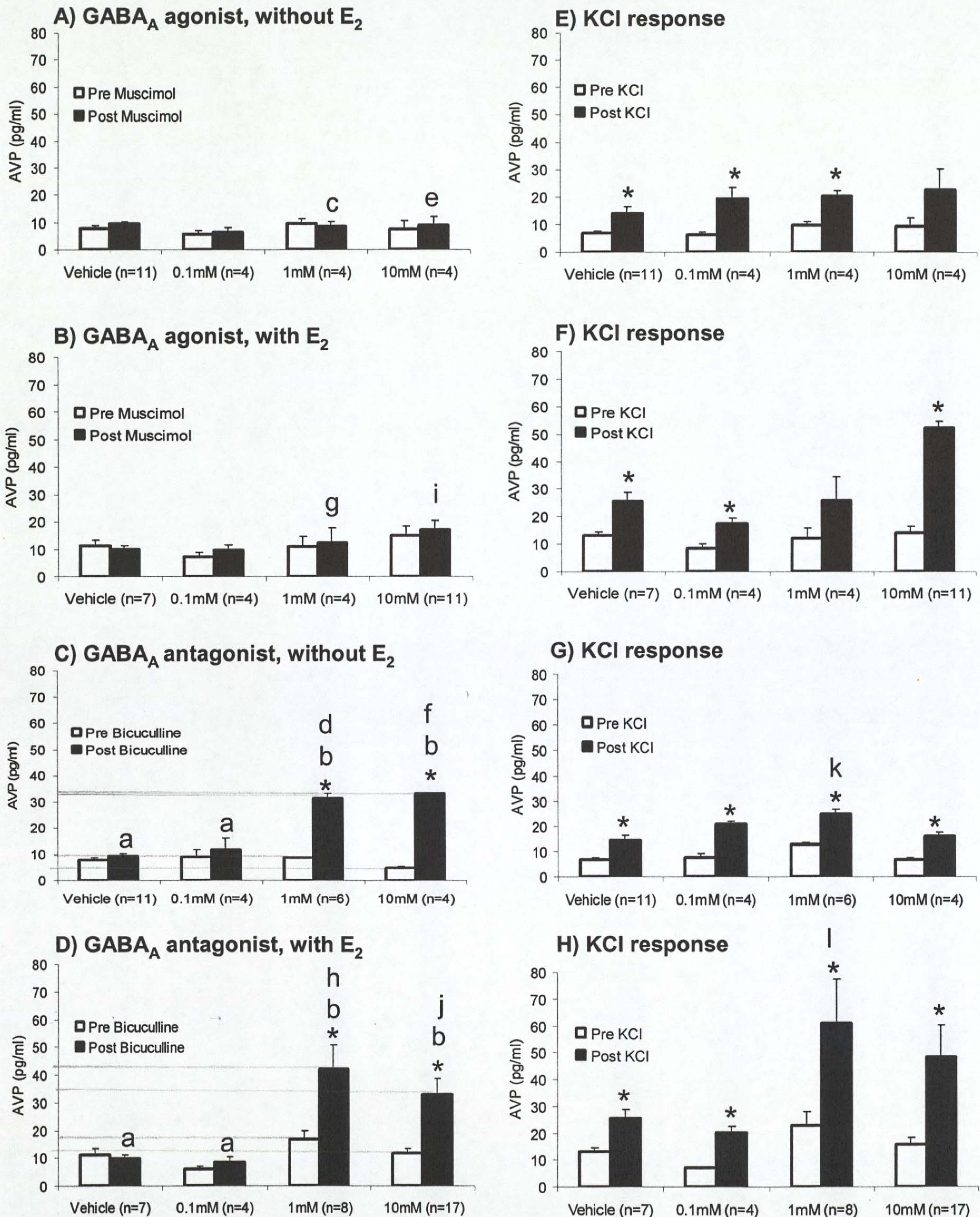


Fig 4.1 Effects of various doses (0.1mM-10mM) of GABA$_A$ agonist (muscimol) or antagonist (bicuculline) and KCl (100mM) challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with media alone or containing oestradiol (E$_2$; 24pg/ml)). The pre- or post-treatment histograms represent average of two 10min fractions collected either immediately pre- or post-treatment. $p<0.05$; from respective pre-treatment values (Asterisks; Paired t-test); between dose groups (a *vs* b) and either with or without E$_2$ (c *vs* d, e *vs* f, g *vs* h, i *vs* j, k *vs* l) by Tukey's pairwise comparison ($p<0.05$). Numbers in brackets represent number of slices.

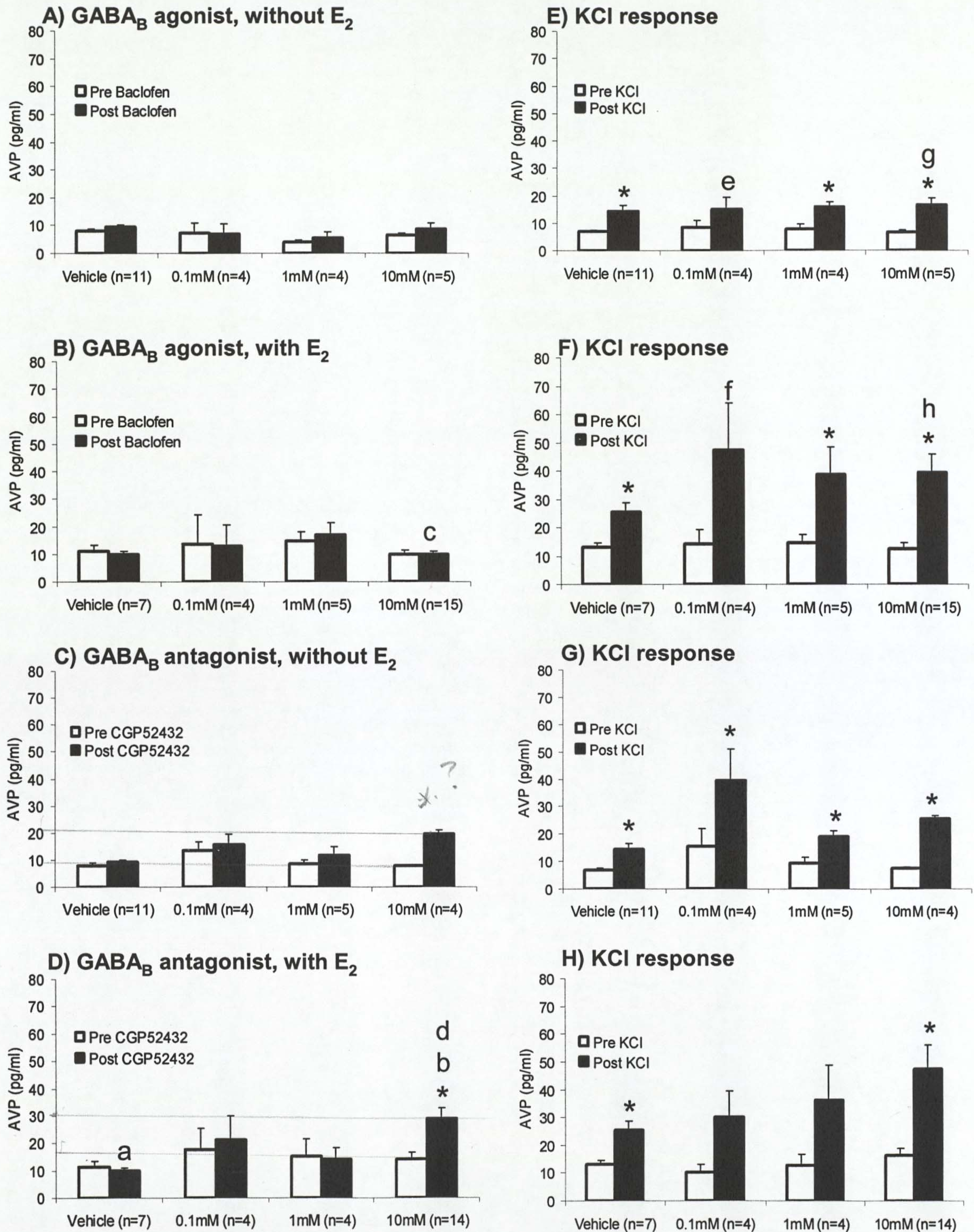


Fig 4.2 Effects of various doses (0.1mM-10mM) of GABA$_B$ agonist (baclofen) or antagonist (CGP52432) and KCl (100mM) challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with media alone or containing oestradiol (E$_2$; 24pg/ml)). The pre- or post-treatment histograms represent average of two 10min fractions collected either immediately pre- or post-treatment. $p<0.05$; from respective pre-treatment values (Asterisks; Paired t-test); between dose groups (a *vs* b) and either with or without E$_2$ (c *vs* d, e *vs* f, g *vs* h) by Tukey's pairwise comparison ($p<0.05$). Numbers in brackets represent number of slices.

GABA$_A$ receptor compounds

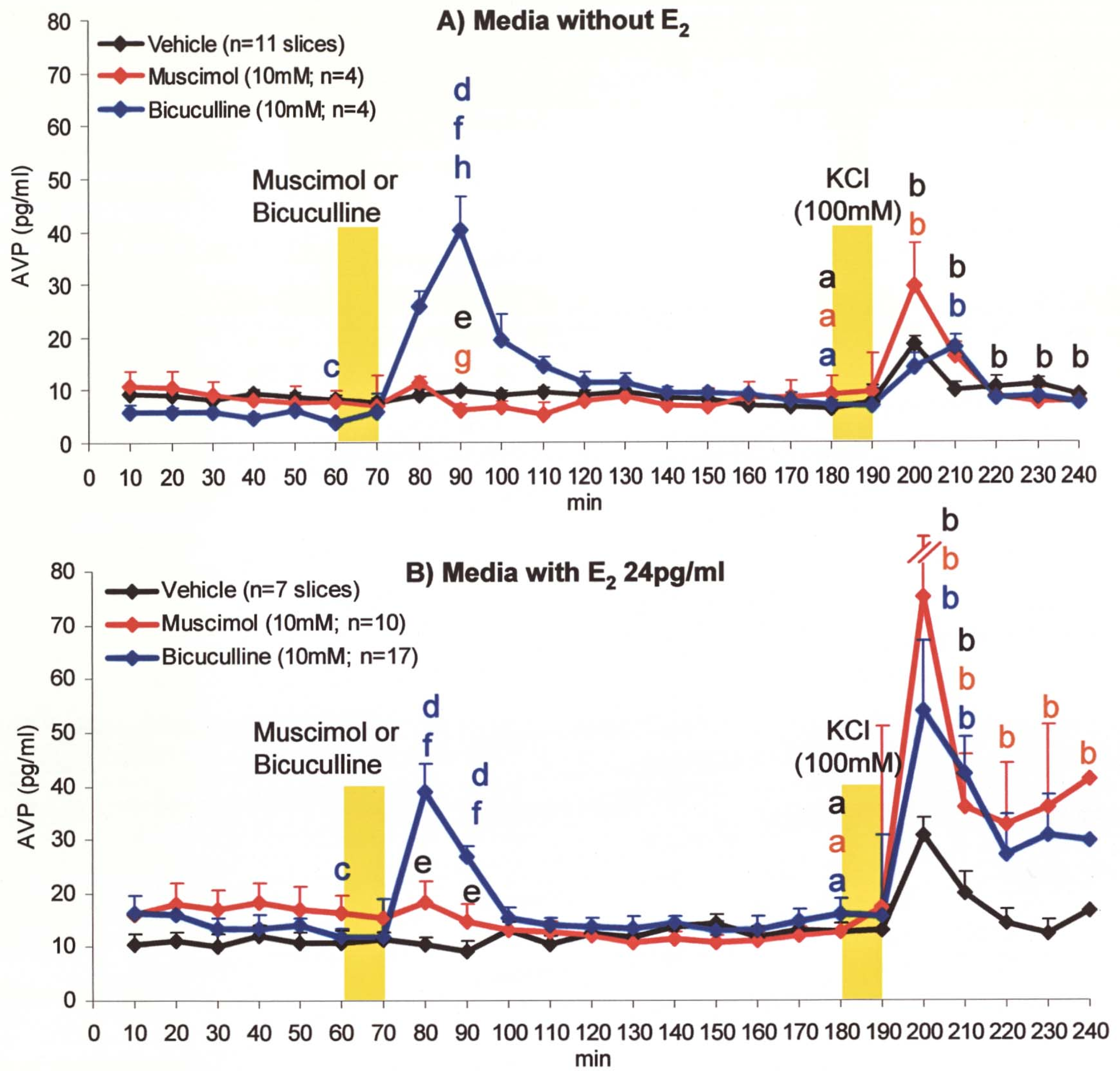


Fig 4.3 Effects of GABA$_A$ agonist (muscimol) or antagonist (bicuculline) and KCl challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with A) media alone or B) containing oestradiol (E_2). $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f; Tukey's pairwise comparison).

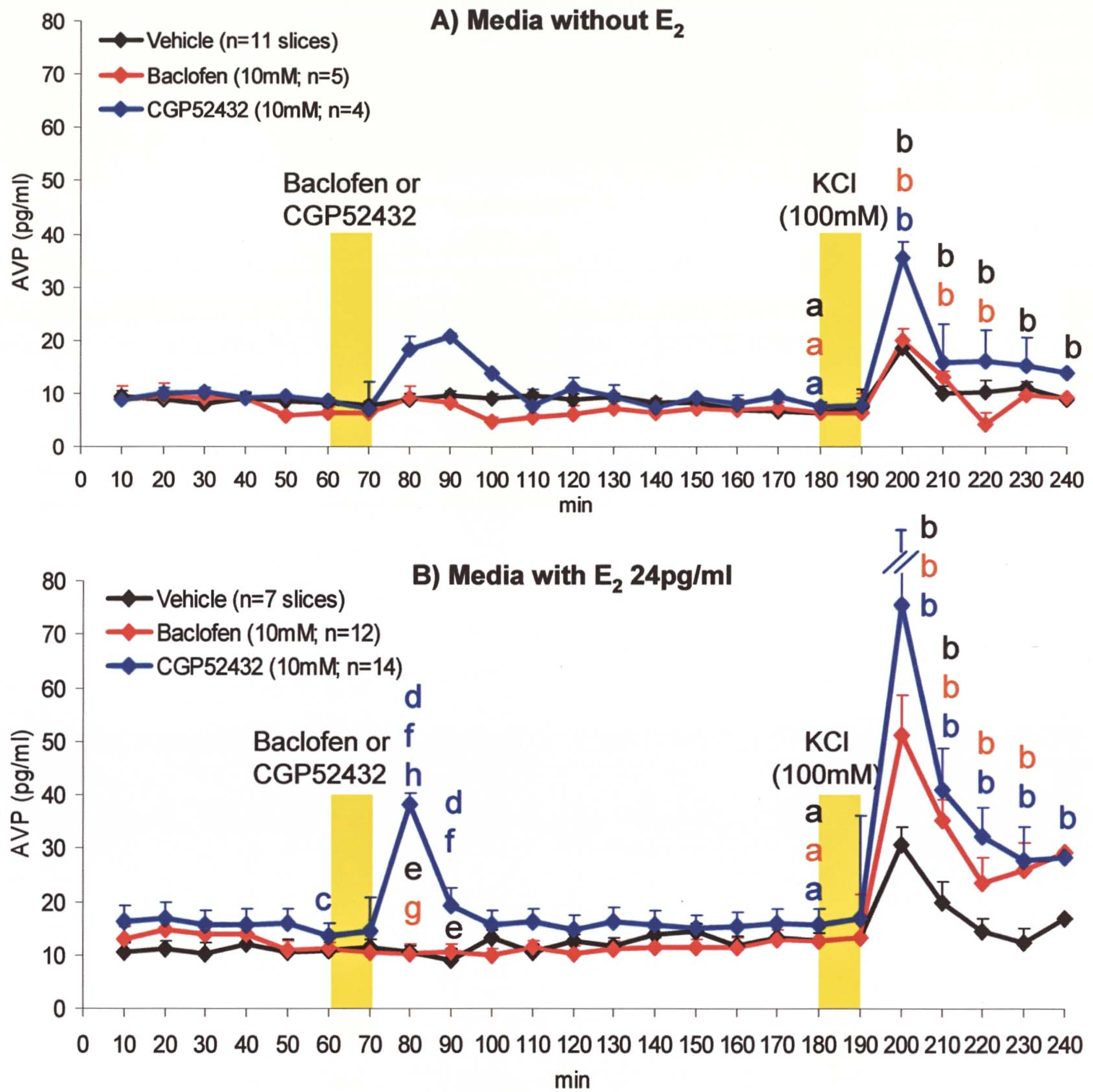
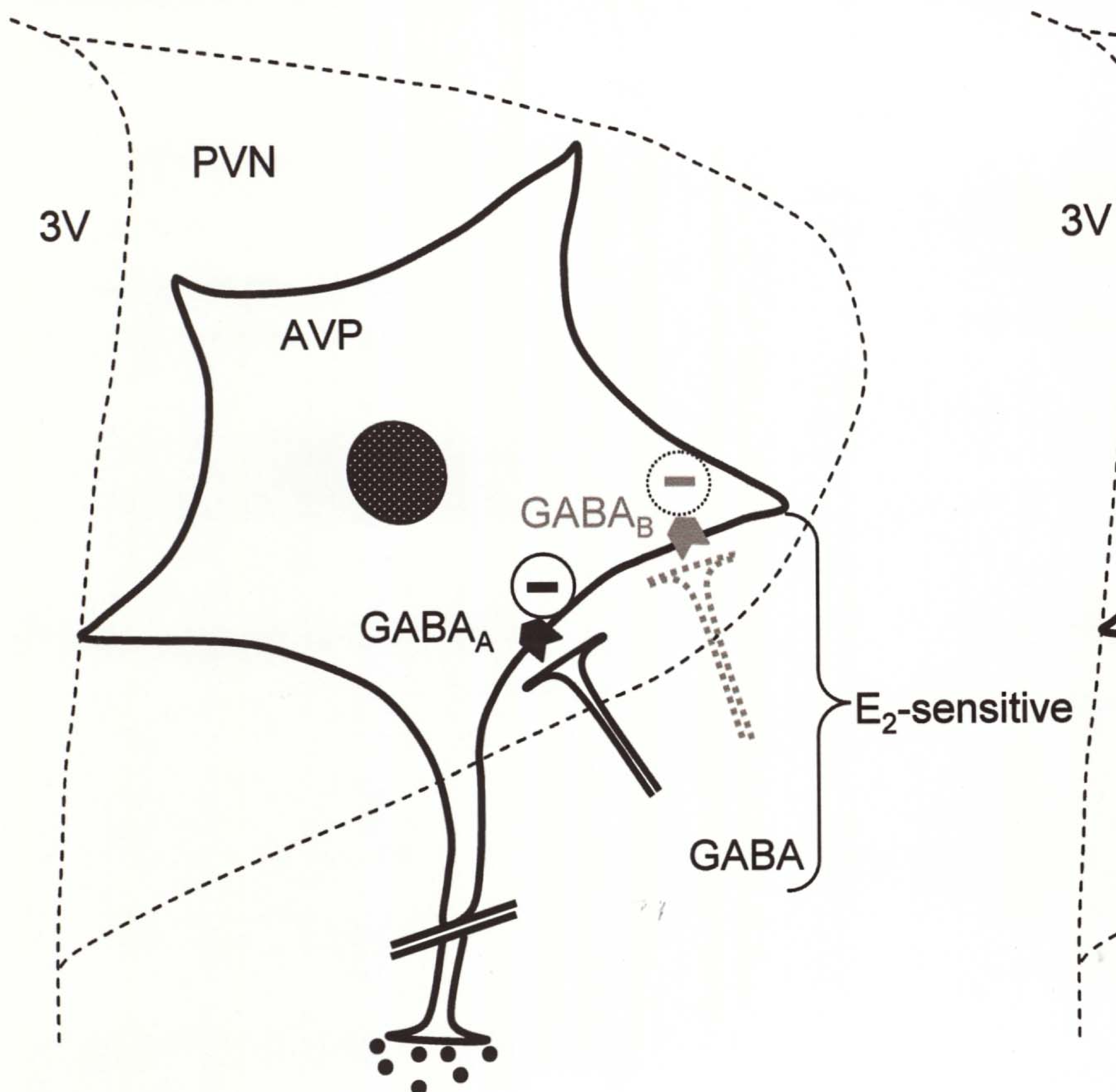


Fig 4.4 Effects of GABA$_B$ agonist (baclofen) or antagonist (CGP52432) and KCl challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with A) media alone or B) containing oestradiol (E_2). $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f, g *vs* h; Tukey's pairwise comparison).

Hypothesis



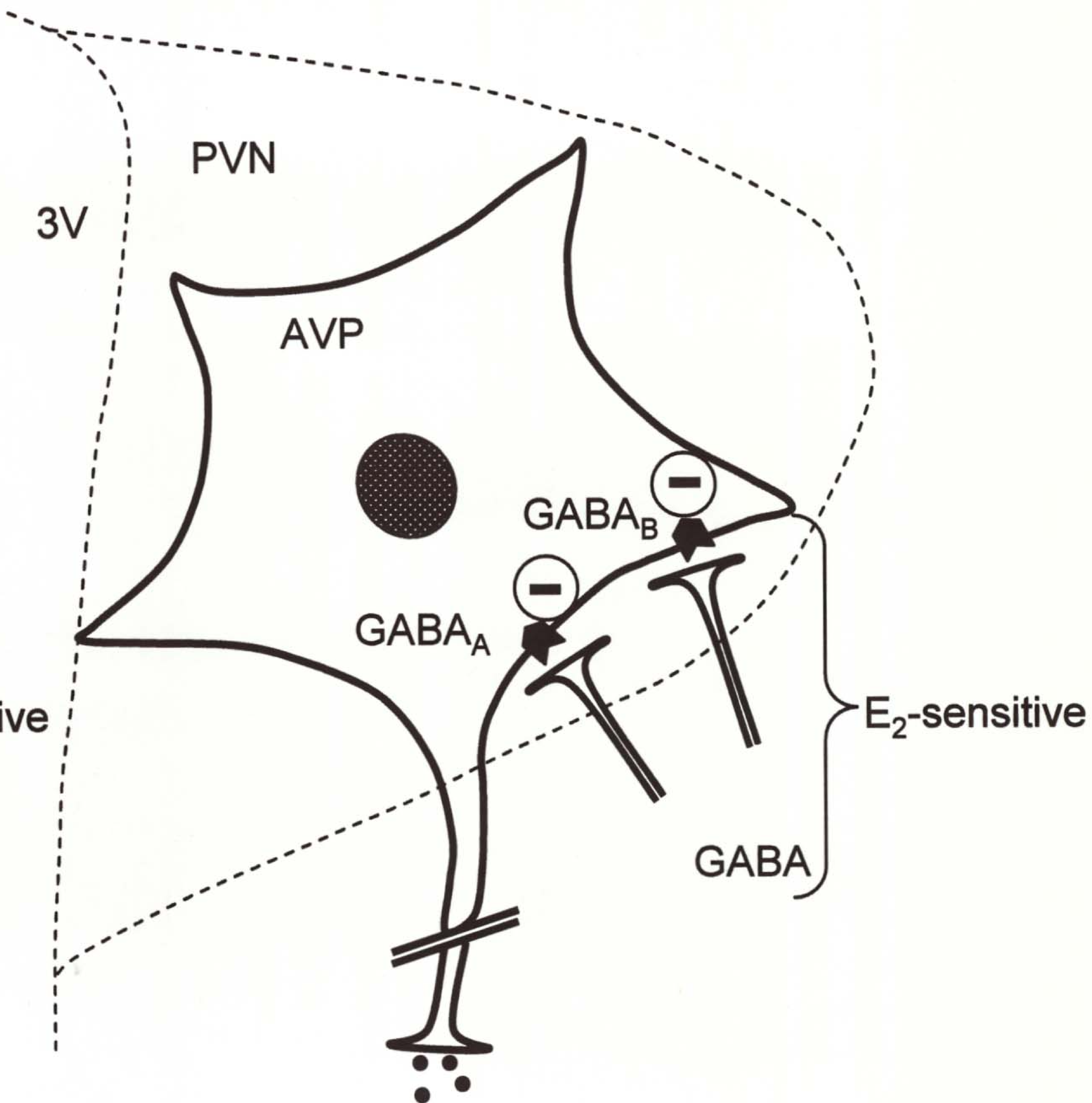


Fig 4.5 Regulation of arginine vasopressin (AVP) neurones by γ-amino butyric acid (GABA) in the absence (A) or the presence of oestradiol (E_2, B). GABA neurones of unknown origin are in the proximity of AVP neurones in the paraventricular nucleus (PVN). Influence of $GABA_A$ receptors is not modulated by the presence of E_2, however, $GABA_B$ receptors inhibit AVP neurones only in the presence of E_2. This suggests stronger inhibitory GABA influence on AVP neurones in the presence of E_2. 3V, Third ventricle

Chapter 5

Noradrenergic control of arginine vasopressin release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

Noradrenergic control of arginine vasopressin release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

ABSTRACT

The present study aims to ascertain the influence of α_1-adrenoreceptors on AVP release *in vitro* and determine whether E_2 modulates the α_1-adrenoreceptor and AVP interaction. Ten minutes after ewe sacrifice, saggital midline hypothalamic slices (from the anterior preoptic area to the mediobasal hypothalamus with the median eminence, 2 mm thick, 2 per sheep) were dissected, placed in oxygenated MEM-α at 4°C and within 2 h were singly perifused at 37°C with oxygenated MEM-α (pH 7.4; flow rate 0.15 ml/min), either with or without E_2 (24 pg/ml). After 4 h equilibration, 10 min fractions were collected for 4 h interposed with10 min exposure at 60 min to a specific α_1-adrenoreceptor agonist or antagonist at various doses (0.1-10 mM). At the end of all perifusions, slices responded to KCl (100 mM) with AVP efflux ($p<0.05$). Release of AVP was enhanced ($p<0.05$) by the α_1-adrenoreceptor agonist (methoxamine 10 mM; No E_2, n=7 perifusion chambers: from 14.3±2.7 to 20.9±3.9, With E_2, n=10: from 10.7±1.2 to 18.4±3.4 pg/ml) or the antagonist (thymoxamine 10 mM; No E_2, n=5: from 9.5±3.1 to 30.4±6.0, With E_2, n=10: from 10.8±0.9 to 39.1±6.3 pg/ml). With the agonist, the response occurred only at 80 min ($p<0.05$) both in the presence and absence of E_2. Whereas, after the antagonist, values were higher ($p<0.05$) throughout the post-treatment period (80-170 min) without E_2, but declined by 150 min in the presence of E_2. Furthermore, the response to the α_1-adrenoreceptor antagonist was greater ($p<0.05$; 90-140 min) than the agonist only in presence of E_2. In conclusion, these results reveal direct α_1-adrenoreceptor-mediated control of the hypothalamic AVP neuronal system which is modulated by E_2.

INTRODUCTION

Arginine vasopressin (AVP) neurones of the hypothalamic paraventricular nucleus (PVN) play an important role in the regulation of hypothalamic-pituitary-

adrenal axis (HPA) through potent stimulatory actions on pituitary adrenocorticotropin hormone (ACTH) secretion in the ewe (Smith *et al.*, 2003a). Parvocellular AVP neurones are the primary source of input to the anterior pituitary, although studies in the rat suggest that paraventricular and supraoptic magnocellular AVP neurones also contribute.

Higher in the brain, noradrenergic pathways originating from the brainstem, particularly the nucleus of solitary tract (NTS) and ventrolateral medulla (VLM), also convey stress-related inputs to the PVN in the rat (Cunningham & Sawchenko, 1988; Ericsson *et al.*, 1994). Extensive data regarding the interaction between noradrenergic inputs and the HPA axis are not available in the ewe. Intracerebroventricular administration of noradrenaline stimulates cortisol release in sheep (Liu *et al.*, 1991), and we recently observed close contacts between noradrenergic terminals and AVP cell bodies in the PVN of the ewe (chapter 9). Several lines of evidence in other species suggest that such noradrenergic inputs play a critical role in controlling the release of AVP. There are direct noradrenergic synapses on AVP neurones in the PVN, and stimulation of the NTS and VLM in the brainstem excites AVP neurones in both the PVN and the supraoptic nucleus (SON) (Day & Renaud, 1984; Kim *et al.*, 1989; Ginsberg *et al.*, 1994; Michaloudi *et al.*, 1997). The effect of noradrenaline may be highly site-specific within the hypothalamus because administration in areas other than the PVN and SON has little or no effect on AVP release (Leibowitz *et al.*, 1990). However, noradrenaline applied to rat hypothalami *in vitro*, or by intracerebroventricular injection, stimulates AVP release (Benetos *et al.*, 1986; Hillhouse & Milton, 1989). Many α_1-adrenoreceptors are present on AVP neurones in the PVN, and the application of α_1-adrenoreceptor agonists to rat hypothalamic explants provokes a depolarization in magnocellular neurones and thus AVP release (Randle *et al.*, 1986; Cummings & Seybold, 1988; Daftary *et al.*, 1998). These observations imply that noradrenaline regulates AVP neurones through α_1-adrenoreceptors directly within the hypothalamus.

Meanwhile, *in vivo* oestradiol (E_2) augments the suppressive effects of HPA activity on the hypothalamic-pituitary-gonadal (HPG) axis, possibly through an effect on AVP neurones (Chen *et al.*, 1992; Cagampang *et al.*, 1997). In the rat and monkey,

AVP has an inverse relationship with the HPG axis by suppressing luteinising hormone (LH) release (Chen *et al.*, 1992; Muret *et al.*, 1992). Furthermore, there is growing evidence that E_2 modulates the AVP neurosecretory system. Intracerebroventricular injection of high doses of AVP suppresses LH secretion only in E_2 replaced ovariectomized (OVX) rats, with no effect in the absence of E_2 (Cates *et al.*, 1999). A significant increase in AVP concentrations in the hypothalamus occurs after E_2 treatment in sheep (Wood *et al.*, 2001), and we have recently demonstrated that E_2 stimulates AVP release from ewe hypothalamic slices *in vitro* (chapter 3).

Thus, it is hypothesised that α_1-adrenoreceptors may participate in the stimulatory effects of E_2 on HPA function, as E_2 alters α-adrenoreceptor binding in the hypothalamus, increases the number of α_1-adrenoreceptors and regulates α_1-adrenoreceptor responses of hypothalamic neurones in the rat and guinea pig (Johnson *et al.*, 1988; Condon *et al.*, 1989; Petitti & Etgen, 1990; Viau & Meaney, 2004). To clarify the direct effects of E_2 on noradrenergic control of AVP release in the ewe, we extended our *in vitro* perifusion system to examine the AVP responses of hypothalamic slices during exposure to α_1-adrenoreceptor compounds in presence or absence of E_2. An *in vitro* approach was essential to avoid any confounding effects of α_1-adrenoreceptor compounds or E_2 on other body systems.

MATERIALS AND METHODS

In vitro perifusion

The perifusion was performed as described elsewhere (chapter 2). After a 4 h equilibration period, medium effluent fractions were collected for 4 h.

Treatments including α_1-adrenoreceptor agonist/antagonist dose response curves

Hypothalamic slices were perifused with media either with or without E_2 (24 pg/ml, Sigma-Aldrich). After 60 min effluent collection, each slice was treated for 10 min with vehicle (MEM-α, without E_2, n=11 perifusion chambers; with E_2, n=7), or increasing doses of an α_1-adrenoreceptor agonist (methoxamine, Sigma-Aldrich; without E_2, 0.1 mM, n=5; 1 mM, n=7; 10 mM, n=7; with E_2, 0.1 mM, n=4; 1 mM,

n=4; 10 mM, n=7) or antagonist (thymoxamine, Parke-Davis, UK; without E_2, 0.1 mM, n=4; 1 mM, n=4; 10 mM, n=10; with E_2, 0.1 mM, n=4; 1 mM, n=4; 10 mM, n=10). At the end of the perifusion, all slices were exposed to a KCl (100 mM) challenge for 10 min after 180 min of effluent collection.

AVP assay

AVP was measured using radioimmunoassay (chapter 2). Sensitivity of the assay was 3.9 pg/ml. Inter- and intra-assay coefficients of variation were 8.1% and 5.9%, respectively.

Statistical analysis

Basal release, with or without E_2, was defined as the mean AVP release in effluent fractions collected for 60 min prior to start of α_1-adrenoreceptor treatment. For dose response studies (Fig 5.1A-D), pre- or post-treatment (methoxamine or thymoxamine) values were calculated by averaging the AVP concentrations of individual chambers at 60 and 70 min for pre-treatment, and at 80 and 90 min for post-treatment. Values for two time points after treatments were averaged as the mean time of maximum response was variable between chambers. Similarly, pre- and post-KCl (Fig. 5.1E-H) concentrations of AVP were calculated by averaging the 180 and 190, or 200 and 210 min fractions, respectively.

Statistical analysis was performed as described previously (chapter 4).

RESULTS

The mean basal (10-60 min) AVP release by hypothalamic slices perifused with medium containing E_2 was higher ($p<0.05$, 10.8±0.1 pg/ml, n=23) than slices perifused with medium containing no E_2 (9.6±0.1 pg/ml, n=27).

Dose responses: Comparing pre- and post-treatment AVP concentrations within each treatment, there was no effect of vehicle or low doses (0.1-1 mM) of methoxamine or thymoxamine, with (Figs 5.1B, 5.1D) or without E_2 (Figs 5.1A, 5.1C). However, after exposure to 10 mM methoxamine or thymoxamine, more AVP

was released compared to pre-treatment values, both in the presence or absence of E_2 (asterisks, Figs 5.1A-D).

After methoxamine treatments, the peak response to 1 mM was greater than after vehicle but only in slices perifused without E_2 (Fig 5.1A; a *versus* b). After thymoxamine with or without E_2, the response to 10 mM was greater than that to vehicle or 0.1 mM (Figs 5.1C, 5.1D; a *versus* b).

Furthermore, the release of AVP after 10 mM thymoxamine was greater than 10 mM methoxamine but only in the presence of E_2 (Figs 5.1B, 5.1D; c *versus* d).

Figure 5.2 emphasizes the prolonged release patterns of AVP from hypothalamic slices during the post-treatment period (80-170 min) following exposure to 10 mM methoxamine or thymoxamine in presence or absence of E_2. The maximum stimulatory response to methoxamine occurred only at 80 min both with and without E_2. In contrast, the response to thymoxamine remained persistently high till 150 or 170 min, in the presence or absence of E_2 respectively. However, the response to thymoxamine was greater from 90 to 140 min than methoxamine only in presence of E_2 (Fig 5.2B).

As illustrated in Figs 5.1E-H and in Fig 5.2, hypothalamic slices responded similarly to KCl (100 mM) with AVP efflux in vehicle and α_1-adrenoreceptor compound-treated groups indicating that the slices were viable and prior compound exposure had no effect.

DISCUSSION

The present study shows for the first time that in the ewe α_1-adrenoreceptors have dual stimulatory and inhibitory control of AVP release within the hypothalamus although the stimulatory effect of the agonist is much lower in the presence of E_2. The significance of this dual α_1-adrenoreceptor-mediated action on AVP release is not clear. Perhaps the most reasonable explanation is that AVP neurones receive inputs from both stimulatory and inhibitory interneurones and final AVP release is determined by the balance of net inhibition/disinhibition. In support of this hypothesis, excitatory noradrenergic/glutamatergic and inhibitory GABA neurones synapse with

AVP neurones in the rat, so activation of α_1-adrenoreceptors in these interneurones will stimulate or inhibit AVP release, respectively (Theodosis *et al.*, 1986; Ginsberg *et al.*, 1994; El Majdoubi *et al.*, 1996; Daftary *et al.*, 1998; Han *et al.*, 2002; Boudaba *et al.*, 2003; Chong *et al.*, 2004). As the present results indicate an influence of E_2 on the function of α_1-adrenoreceptors to modulate AVP release, any dual effects are unlikely to be a result of neurotoxicity. Overall, evidence suggests that indirect routes of noradrenergic action may be important to modulate AVP release.

The α_1-adrenoreceptor-mediated stimulation of AVP release extends previous *in vivo* and *in vitro* observations from other species in which α_1-adrenoreceptor activation excites magnocellular neurones to induce AVP release (Benetos *et al.*, 1986; Randle *et al.*, 1986; Shioda *et al.*, 1997; Daftary *et al.*, 1998; Boudaba *et al.*, 2003). But it remains intriguing that AVP release was stimulated by both the α_1-adrenoreceptor agonist and antagonists and the response to the antagonist was greater. As we have tested a wide range of doses, we are confident that there is strong inhibitory, rather than stimulatory, control of AVP neurones in this *in vitro* system. In support, recent electrophysiological studies reported α_1-adrenoreceptor-mediated inhibition of magnocellular and parvocellular PVN neurones (Han *et al.*, 2002; Chong *et al.*, 2004). Similarly, combined *in situ* hybridization and immunohistochemistry of *in vitro* incubated hypothalamic slices concluded that noradrenaline inhibits AVP release from PVN and SON neurones via α_1- and α_2-adrenoreceptors in the rat (Iamova *et al.*, 2002). The prolonged influence of influence of α_1-adrenoreceptor compounds on release of AVP from the hypothalamic slices is not surprising and could be due to prolonged occupancy of the α_1-adrenoreceptors within the hypothalamus. Previous, *in vitro* perifusion studies using bovine hypothalamic slices have observed similar effects of α_2-adrenoreceptor or dopamine receptor compounds on growth hormone release for 60-80 min after the end of exposure (West *et al.*, 1997; McMahon *et al.*, 2001b).

Basal release of AVP from the hypothalamic slices was augmented by E_2 confirming our previous *in vitro* data (chapter 3) in agreement with *in vivo* studies in sheep (Wood *et al.*, 2001). However, the presence or absence of E_2 did not affect the initial responses to the α_1-adrenoreceptor agonist or antagonist in the present study.

Following earlier observations (chapter 4), it is hypothesised that noradrenergic systems interacts with GABA systems to suppress AVP secretion in the presence of E_2. In the rat, $GABA_A$ receptor activation inhibits parvocellular PVN neurones and such tonic inhibition is enhanced by activation of α_1-adrenoreceptors on GABA neurones (Han *et al.*, 2002). In the ewe, E_2 potentiates inhibition of AVP through $GABA_B$ (but not $GABA_A$) receptors resulting overall in additional inhibition of AVP neurones in presence of E_2 through the combined influence of both $GABA_{A \text{ and } B}$ receptors (chapter 4). Taken together, all this evidence explains the α_1-adrenoreceptor-mediated suppression of AVP in the presence of E_2 (Fig 5.3). In the presence of E_2, the activation of α_1-adrenoreceptors on GABA neurones becomes predominantly inhibitory overcoming any stimulatory effects via α_1-adrenoreceptors on the AVP or other interneurones. After initial antagonism of α_1-adrenoreceptors, the marked increase in AVP in the presence of E_2 arises by removal of the strong GABA inhibition of AVP neurones. Later, the inhibitory GABA influence maintained by E_2 becomes prevalent resulting in an early decline in AVP concentrations. In the absence of E_2, GABA influence is weak and thus AVP remains elevated. Overall, this suggests that in an E_2 milieu, α_1-adrenoreceptor-mediated actions on AVP secretion are modulated by GABA.

There is an alternate explanation for the greater response to the antagonist than the agonist in the presence of E_2. This is based upon existing circumstantial evidence in the rat literature. Peripheral administration of prazosin, another α_1-adrenoreceptor antagonist, blocks accumulation of E_2-receptors within the preoptic area and mediobasal hypothalamus (Montemayor *et al.*, 1990). Prazosin also antagonizes the stimulatory effect of E_2 to increase AVP content in the median eminence (Viau & Meaney, 2004). Similarly in the present study, the α_1-adrenoreceptor antagonist suppresses AVP release in a high E_2 environment. However, *in vivo*, the stimulatory effect of E_2 on AVP expression through α_1-adrenoreceptors occurs only with low concentrations of E_2 and progesterone (Viau & Meaney, 2004). The present study used high E_2 concentrations without progesterone; clearly further *in vitro* studies with varying ovarian steroid concentrations in the perifusion media are warranted.

Apart from the effects of E_2 via α_1-adrenoreceptors on AVP neurones in the hypothalamus, there may be extra hypothalamic site(s), not present in these slices, of E_2 action to modulate noradrenergic influence on AVP neurones. In the PVN, AVP neurones are innervated by noradrenergic projections from the lower brainstem, where E_2-receptors colocalise with noradrenergic cells and are activated in response to stressors and peripheral E_2 administration (Cunningham & Sawchenko, 1988; Gaillet *et al.*, 1991; Ginsberg *et al.*, 1994; Scott *et al.*, 1999; Rawson *et al.*, 2001). This indirect external influence through E_2 sensitive noradrenergic projections to the PVN in relation to potentiation of the stress responses *in vivo* requires further detailed investigations.

Finally, in contrast to the observed inhibitory influence through α_1-adrenoreceptors, early investigations suggested that the inhibitory action of adrenoreceptors on AVP release is principally mediated via subtype-β receptors (Day *et al.*, 1985; Takano *et al.*, 1989). This could be explained by glucocorticoids modifying the function of adrenoreceptors. Glucocorticoids were not included in the present perifusion medium although a few studies suggest these steroids may play a role in the shift of adrenoreceptor subtype function. With glucocorticoids, noradrenaline stimulates CRH release but under glucocorticoid-free conditions, noradrenaline strongly inhibits CRH secretion from rat cultured hypothalamic slices through α_1- and α_2/β-adrenoreceptors, respectively (Szafarczyk *et al.*, 1995; Feuvrier *et al.*, 1998). A similar interaction for AVP neurones may exist as both AVP and CRH are regulated by glucocorticoids and noradrenaline in the rat (Itoi *et al.*, 1987; Hillhouse & Milton, 1989).

In conclusion, we have demonstrated an α_1-adrenoreceptor-mediated noradrenergic control of AVP neurones within the isolated ewe hypothalamus. However, further investigations are required to elucidate the precise interactions of steroids (E_2, progesterone and glucocorticoids) on AVP neurones via the noradrenergic system. Interactions with other interneurones especially GABA may modulate noradrenergic influence on AVP neurones in the ewe.

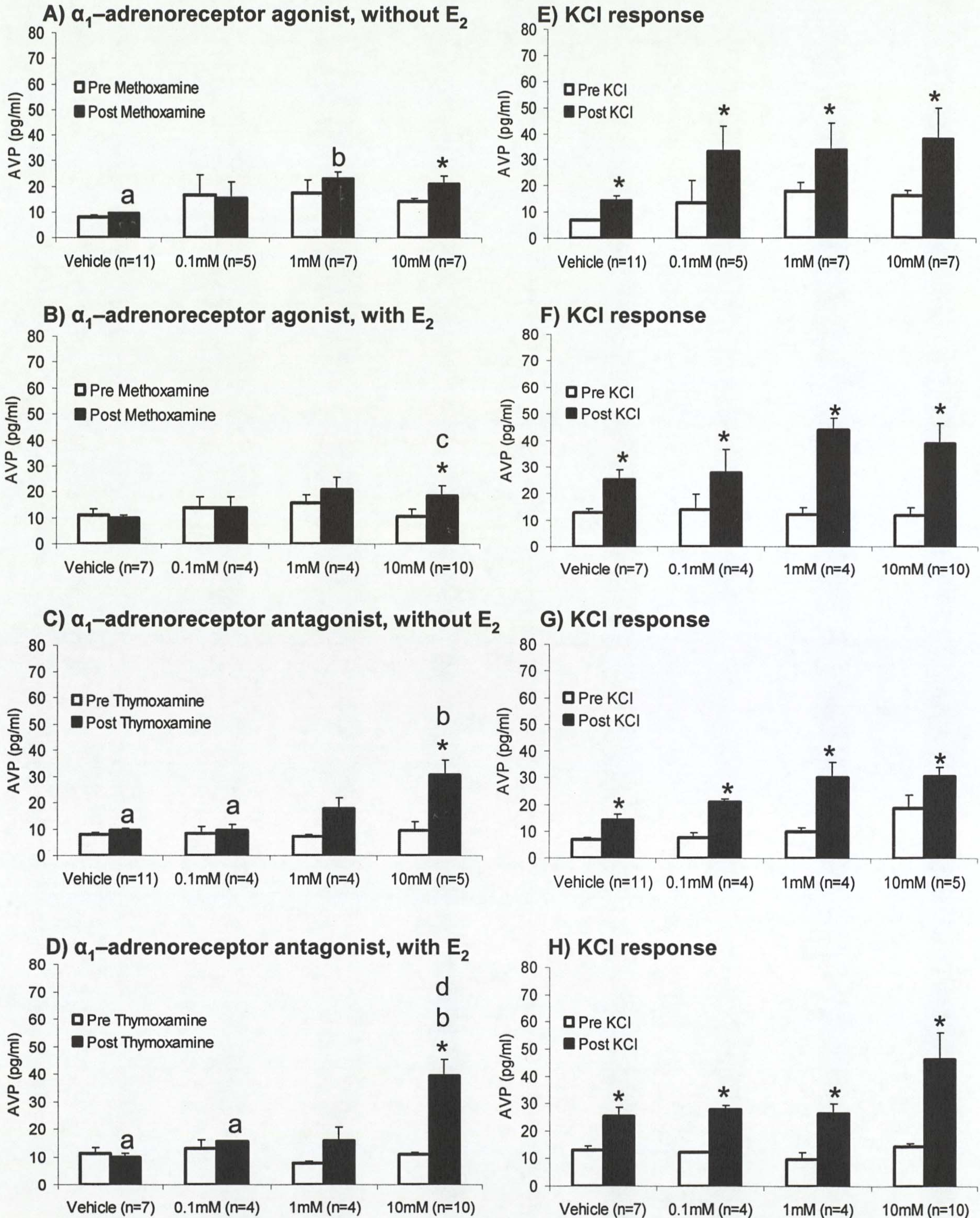


Fig 5.1 Effects of various doses (0.1 mM-10 mM) of α_1–adrenoreceptor agonist (methoxamine) or antagonist (thymoxamine) and KCl (100 mM) challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with media alone or containing oestradiol (E_2; 24 pg/ml). The pre- or post-treatment histograms represent average of two 10min fractions collected either immediately pre- or post-treatment. $p<0.05$; from respective pre-treatment values (Asterisks; Paired t-test), between dose/drug groups (a *vs* b, c *vs* d; Tukey's pairwise comparison). Numbers in brackets represent number of slices.

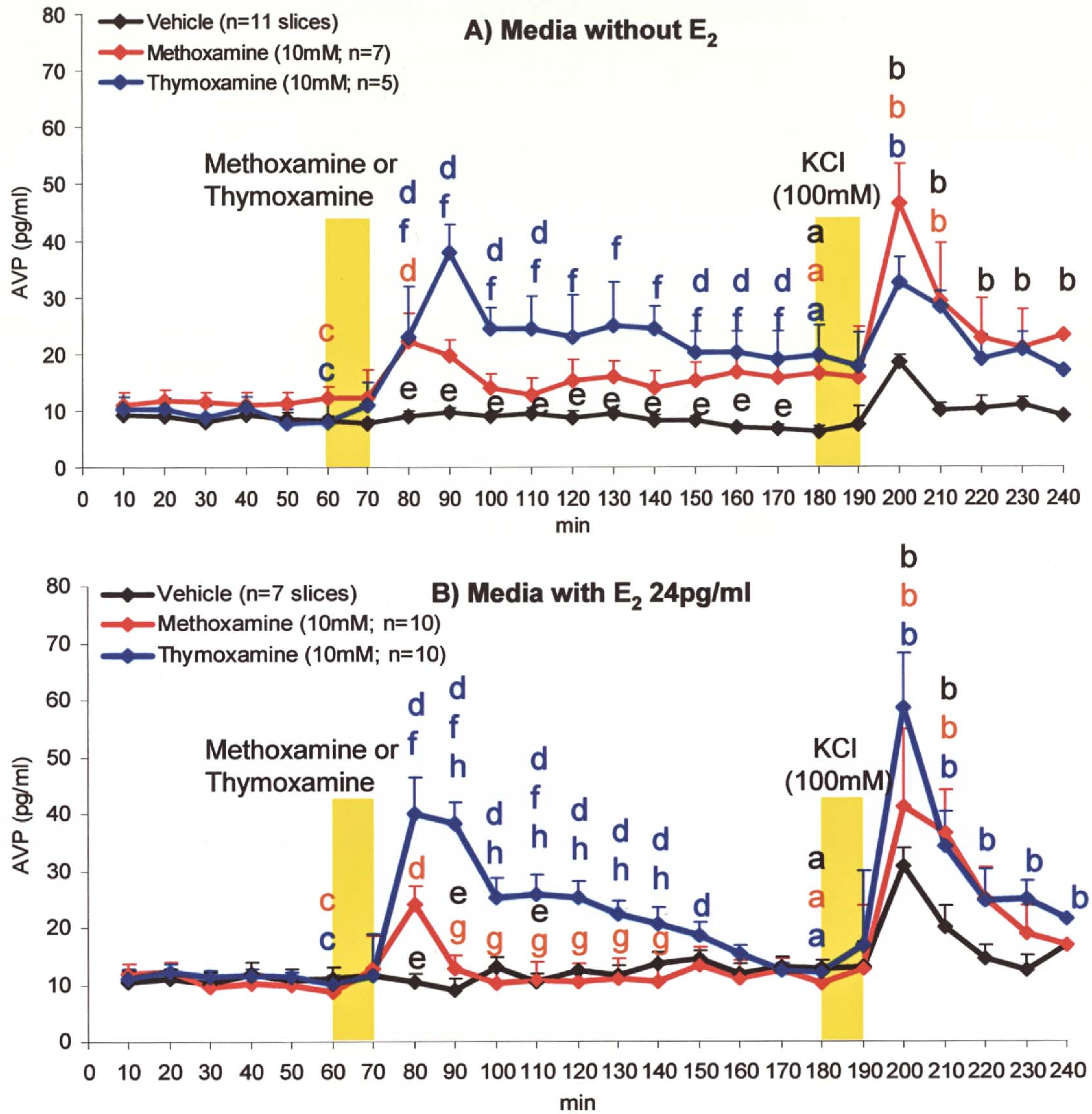
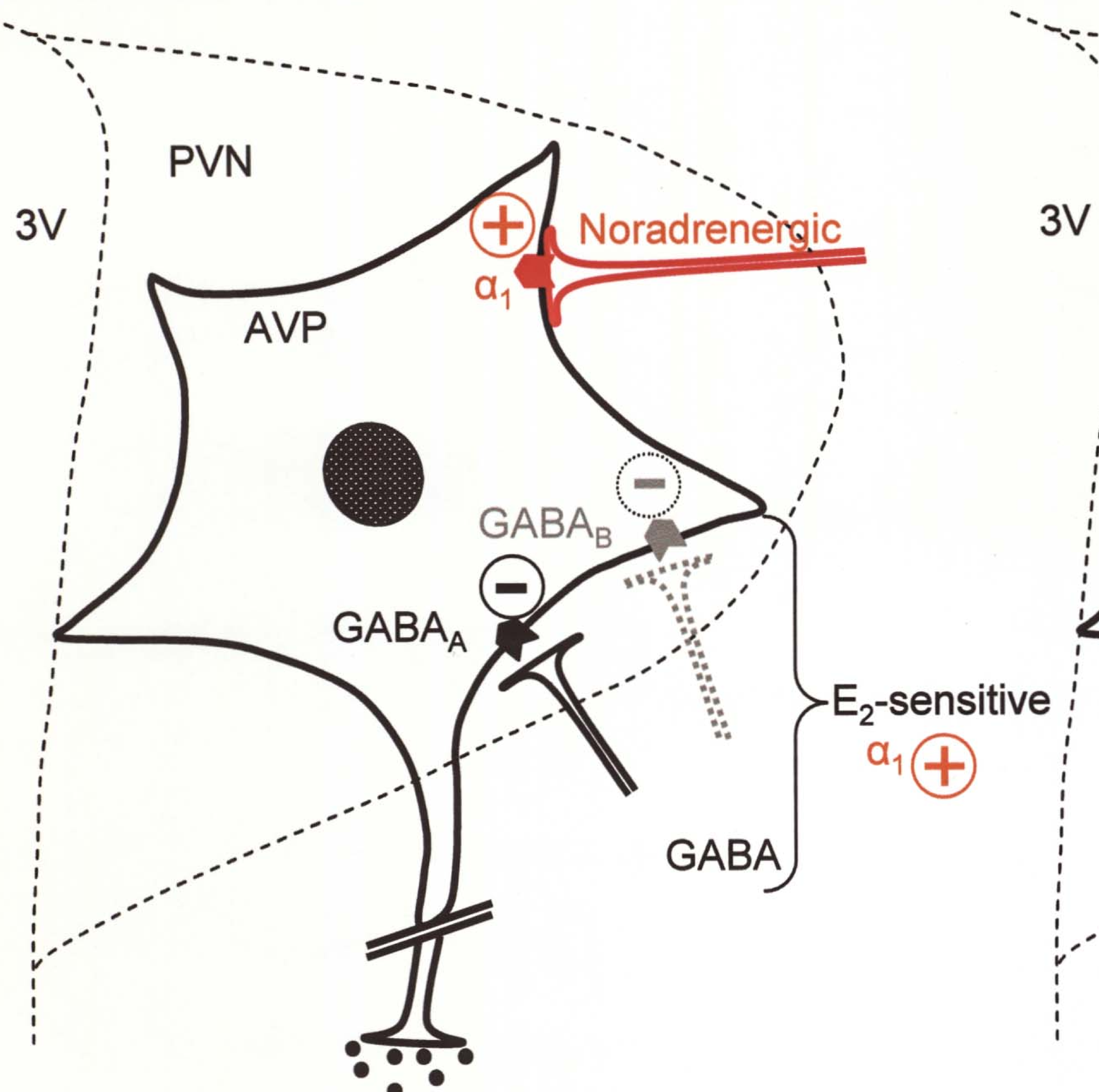


Fig 5.2 Effects of α_1-adrenoreceptor agonist (methoxamine) or antagonist (thymoxamine) and KCl challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with A) media alone or B) containing oestradiol (E_2). $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f, g *vs* h; Tukey's pairwise comparison).

Hypothesis



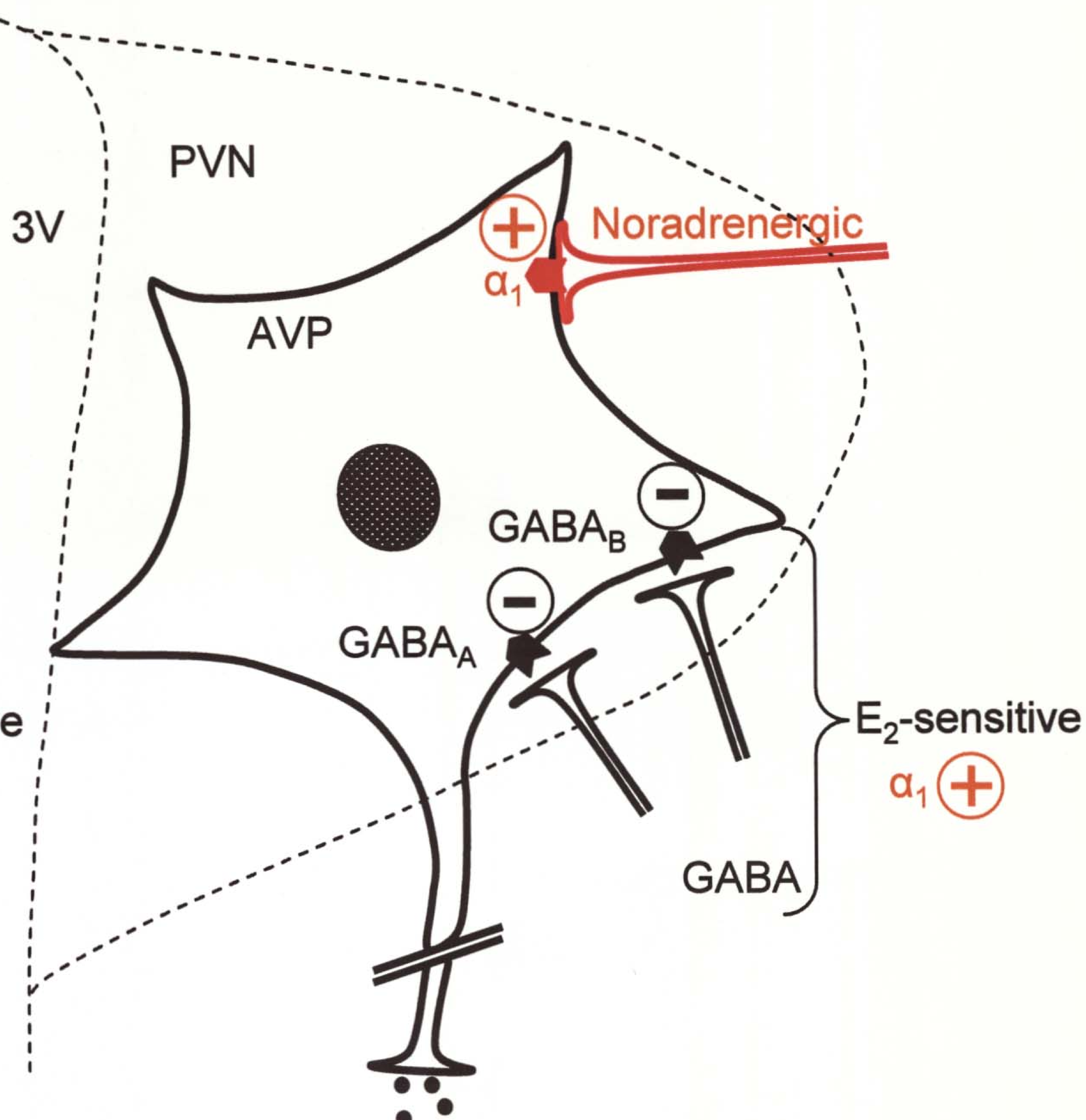


Fig 5.3 Regulation of arginine vasopressin (AVP) neurones by noradrenaline in the absence (A) or the presence of oestradiol (E_2, B). Noradrenergic terminals of unknown origin are in close contact with AVP neurones in the paraventricular nucleus (PVN). In the absence of E_2 (A), α_1-adrenoreceptors present on the GABA neurones release GABA which predominantly acts through only $GABA_A$ receptors, however, activation of α_1-adrenoreceptors on the AVP neurones may overcome this GABA inhibitory effect leading to net stimulatory response. But, in the presence of E_2 (B), α_1-adrenoreceptor induced release of GABA occurs via both $GABA_{A \text{ and } B}$ receptors overcoming the stimulatory influence of α_1-adrenoreceptors, ultimately leading to net inhibition of AVP secretion. 3V, Third ventricle

Chapter 6

Attempts to separate α_1-adrenoreceptor or GABA receptor compounds from hypothalamic perifusate samples containing GnRH

Attempts to separate α_1-adrenoreceptor or GABA receptor compounds from hypothalamic perifusate samples containing GnRH

ABSTRACT

A non-polar SPE method was used to attempt to separate α_1-adrenoreceptor (methoxamine) or GABA receptor (bicuculline) compounds and GnRH from perifusion media. Both EC and NEC and C_{18} columns were tested in-parallel using methanol or acetonitrile as organic elution solvent. Different concentrations of organic elution solvent in water titrated to a range of pH were evaluated. Methoxamine and bicuculline eluted earlier compared to GnRH from C_{18} EC columns when eluted with 40-45% methanol (pH 2.5-3.0), but due to wide range of the peaks separation was not reproducible and accurate enough to be used routinely. In conclusion, due to minimal differences between the non-polar character of GnRH, α_1-adrenoreceptor and GABA receptor compounds, the SPE method attempted was insufficient and SPE followed by HPLC may be more appropriate.

INTRODUCTION

During *in vitro* perifusion experiments with hypothalamic slices (chapter 7 and 8), α_1-adrenoreceptor or GABA receptor compounds were used to evaluate their effects on GnRH release. After exposure to slices, GnRH released and the compounds were collected in the same perifusate fractions. When these samples were assayed for GnRH, some of the compounds (e.g. methoxamine, bicuculline) interacted with the GnRH assay to give a false increase in immunoreactive GnRH concentrations. Therefore, it was necessary to attempt to separate these compounds from GnRH in the perifusate fractions to allow the true change in GnRH concentrations as a result of methoxamine or bicuculline action on the hypothalamic slices to be measured.

A non-polar solid phase extraction (SPE) method (Hennion, 1999) was used to separate GnRH from methoxamine or bicuculline present in the perifusate samples. SPE methods are routinely used for preparation of biological samples i.e. sample

clean-up, removal of biological matrices interferences, before applying the samples directly to high pressure liquid chromatography (HPLC), as the inevitable analytes present in biological matrices can cause HPLC column clogging and analyte ion suppression due to the presence of proteins, lipids, salts and other endogenous material (Constanzer *et al.*, 1997). However, we attempted a cost-effective SPE method alone to separate methoxamine or bicuculline from the perifusate samples containing GnRH. Previously, in our lab SPE system has been successfully used to separate arginine vasopressin and corticotrophin releasing hormones from the same portal plasma sample to allow radioimmunoassay of each compound (Smith *et al.*, 2003a). In other labs, the isolation of some β-blocking compounds from plasma by SPE, using either an Extrelut-1 column or disposable extraction cartridges packed with octadecyl- or cyanopropyl-bonded silica, has been performed (Devi *et al.*, 1988; Musch & Massart, 1988).

The mechanism of SPE is based on hydrophobic interactions of both, the analytes of interest (GnRH) and interfering compound (methoxamine or bicuculline) with the adsorbent surface of silica columns (Fig 6.1). The adsorbents are formed by bonding a hydrophobic phase to a silica matrix. The hydrocarbon group forming the hydrophobic phase is a linear aliphatic hydrocarbon of two (C_2), eight (C_8) or eighteen (C_{18}) carbon atoms (Hennion, 1999).

The four sequential steps of a typical SPE sequence are (i) conditioning of the silica based sorbent mass in the column, (ii) application of the sample, (iii) rinsing the sample, and (iv) desorption and recovery of the analytes to be separated (Fig 6.1). In brief, in the first step, the solvation or wetting of the sorbent is performed by passing bed volumes of an organic solvent through the silica columns. In the sample loading step, the analytes are then adsorbed on the solid hydrophobic phase while other hydrophilic endogenous compounds pass through the sorbent bed during rinsing. Analytes remain adsorbed until the concentration of the organic eluent reaches a critical concentration to break these hydrophobic interactions and cause desorption. The number of organic modifier molecules required to desorb a specific analyte is very precise, hence desorption takes place within a very narrow window of organic eluent concentration (Geng & Regnier, 1984; Kunitani *et al.*, 1986).

Endcapped (EC) and non-endcapped (NEC) silicas are available. Non-endcapped silicas have an increased number of free silanol groups (negative charged), at the bonded silica surface to provide secondary interactions with solutes so that one of the analytes may be poorly retained in the column due to less secondary interactions, while the other may be more retentive allowing separation of compounds that could not be resolved using EC silicas (Henion *et al.*, 1998). GnRH, methoxamine and bicuculline contain a basic group (amines, positive charge), so it is also possible to utilize ionic interactions between the exposed silanol groups of the non-polar adsorbents and the basic moiety of GnRH and the compounds. These are retained by both a non-polar and ionic-retention mechanism, allowing a more rigorous interference regime to be used to break ionic and hydrophobic interactions. Thus, by changing the pH of the samples and the concentration of an organic eluent, GnRH or the compounds may have different retention patterns on non-endcapped columns.

From the literature, methanol, an organic eluent was found to give high recoveries in most instances (Hubert *et al.*, 1994). Methanol is an effective solvating agent because it can interact with both the silanol groups at the silica surface and the carbon atoms of the bonded alkyl chains. We also compared the elution efficacy of methanol with acetonitrile, another organic eluent.

The important factor with respect to the analyte separation and recovery is the dispensing flow-rate of the fluids on the silica columns. Indeed, at higher elution flow-rates, the recoveries and separation of the analytes decrease drastically because the residence time of the samples in the silica columns is reduced to such an extent that only part of the analytes are adsorbed and can be distributed throughout the solid phase (Hubert *et al.*, 1991; Hubert *et al.*, 1994). Consequently, the minimum flow-rate, which due to gravitational pressure alone, was selected through out the various steps in order to obtain the best possible results.

In the present study, a range of silica columns (C_2, C_8, C_{18}, EC or NEC) were tested over a wide pH range (2.5-11) of the elution conditions. The aim was to identify a method that would allow precise, accurate and repeatable separation of GnRH and interfering compounds (methoxamine and bicuculline) into two elute fractions. However, a method development procedure, which eventually will lead to an

understanding of the relationship between the retention behavior of these analytes with respect to the pH value and the percentage of organic solvent, is a formidable and time-consuming task.

MATERIALS AND METHODS

Flow rate

The flow rate, under gravitational pressure, of fluids (sample or methanol or PB), in the ISOLUTE columns (International Sorbent Technology, Hengoed, Mid-Glamorgan, UK) was 0.5 ml/15 min. No pressure was applied onto the columns to increase the flow rate. After each addition, the fluid was allowed to run through the column until the top of the sorbent bed was just dry before any other compound was added.

Sorbent conditioning

ISOLUTE C_2, C_8 or C_{18} endcapped (EC) or non-endcapped (NEC) columns (100 or 500 mg sorbent mass in 1 or 3 ml column volume), respectively were conditioned with acetonitrile (2 ml) or acidified methanol (2 ml; 80% methanol with 0.1% trifluoroacetic acid) followed by 0.1 M phosphate buffer (PB) pH 7.4 or the same pH as the acidified methanol used depending on experiment.

Sample loading

Samples containing GnRH, methoxamine or bicuculline individually or GnRH with methoxamine or bicuculline were applied depending upon the experiment. In the first experiment, identical aliquots of radioactive I^{125} GnRH were applied to 100 mg sorbent mass C_2, C_8 or C_{18} EC columns. In the second experiment, the same amounts of unlabelled GnRH, methoxamine or bicuculline were applied to 500 mg sorbent mass C_{18} EC or C_{18} NEC columns. After these initial experiments, the same amount of GnRH or GnRH with methoxamine was applied to 500 mg sorbent mass C_{18} EC or C_{18} NEC columns while bicuculline was tested only with the 500 mg sorbent mass C_{18} EC columns. This was due to the requirement to potentially use larger columns to allow

more media to be applied, thus extracting more peptide and increasing the sensitivity of the system. As the elution patterns of methoxamine and bicuculline were similar one compound, methoxamine, was used in most experiments.

Washing

Columns were then rinsed with six aliquots of 2 ml PB. In the initial two experiments, pH of the PB was 7.4,[as used previously in our lab (Smith *et al.*, 2003a)], thereafter pH of the PB was the same as the pH of the methanol used during elution procedures (see tables 6.1-6.3).

GnRH or methoxamine or bicuculline elution

In the first and second experiment, iodinated GnRH, GnRH or methoxamine or bicuculline was eluted by applying to the same column increasing concentrations (0 to 100%, 1.5 ml each) of acetonitrile or methanol. In the second part of the second experiment, GnRH or methoxamine or bicuculline applied to EC or NEC columns were washed with 1.5 ml 30% or 50% methanol, respectively, followed by a final wash with 100% methanol. In subsequent experiments, C_{18} EC or NEC columns containing GnRH alone or mixed with methoxamine or bicuculline were eluted with repeated 0.5 ml aliquots of various concentrations of methanol at a range of pH (see tables 6.1-6.3 for details). At the end, columns were washed with 0.5 ml 100% methanol of the same pH (repeated 1-3 times).

GnRH assay

At the end of extraction, eluates were dried overnight in a vortex evaporator (Speedvac plus concentrator; Savant, Holbrook, NY) at 40°C, and reconstituted in 250 μl assay diluent by vortex mixing. GnRH-like activity of methoxamine, bicuculline or GnRH was measured by RIA described elsewhere (chapter 2). For simplicity, in results and figures, a measurement of eluted methoxamine or bicuculline is termed GnRH-like immunoreactivity while elution of GnRH is termed as GnRH.

RESULTS

Using C_2, C_8 and C_{18} EC columns, I^{125} GnRH was eluted with 30-40% acetonitrile (Fig 6.2A), whereas 25, 35 and 45% methanol eluted I^{125} GnRH from C_2, C_8 and C_{18} EC columns (Fig 6.2B). Comparing EC and NEC C_{18} columns, GnRH started to elute at 40 and 70% methanol, whereas methoxamine or bicuculline, as indicated by GnRH-like activity of these compounds, began to elute at 30 and 50% methanol, respectively (Fig 6.3A). However in subsequent trails 30 or 50% methanol interference wash was not able to elute methoxamine or bicuculline from EC or NEC columns, respectively (Fig 6.3B).

When GnRH and methoxamine were applied together in media acidified to pH 5.0 to C_{18} columns, GnRH and GnRH-like activity of methoxamine appeared to separate at 50 and 55% methanol in EC columns and 60 and 70% methanol in NEC columns (Table 6.1A). But, this was not reliably repeatable as determined by the wide range of the peaks (Table 6.1B). However, methanol at pH 3 and concentration of 45% and 55% when respectively applied to EC and NEC columns appeared to separate methoxamine from GnRH (Table 6.2A). When the experiment was repeated with EC columns, at methanol pH 2.5 and concentration between 35 to 45%, methoxamine appeared to elute first and GnRH in the next fraction (Table 6.2B). Similarly, from EC columns, bicuculline at pH 3.0 and 40% methanol appeared to elute one fraction earlier than GnRH, however with higher methanol concentration or pH, both GnRH and bicuculline started to elute in the same fractions (Table 6.3).

DISCUSSION

With the time available it was not possible to develop a method that reliably separated α_1-adrenoreceptor (methoxamine) and GABA receptor (bicuculline) compounds from GnRH in perifusate samples by an SPE technique. This method employed the concept of manipulating both the pH and the percentage of organic eluent (methanol) in the mobile phase to affect the retention behaviors of both the matrix interference analytes (α_1-adrenoreceptor and GABA receptor compounds) and

the analytes of interest (GnRH). However, the results obtained provide specific information of the retention behavior of these analytes with respect to the pH value and the percentage of organic eluent which could in future enable the design of an optimized, specific acid/base/organic washing SPE method, resulting in effective removal of matrix interferences.

Systematically testing different columns is the only practical way of determining which column will give the best resolution (Tempst *et al.*, 1986). The C_{18} columns were best to retain GnRH, possibly due to the more hydrophobic surface provided by the long carbon chains in C_{18} columns which takes higher concentration of methanol to break the hydrophobic interactions. Also, the smallest peptides are often best separated on small pore (55A°) C_{18} columns with greater retention, though C_8 columns with slightly shorter retention are recommended for proteins larger than 5,000 daltons. In the present study methanol gave better retention than acetonitrile however the literature does not suggest any difference between the use of acetonitrile and methanol for peptide separation (Hubert *et al.*, 1994; Hennion, 1999). We initially used 100 mg sorbent mass in the column with latter trials using 500 mg. The adsorption/desorption of peptides responsible for their separation takes place almost entirely near the top of the column (Geng & Regnier, 1984). Therefore, column length depending upon the amount of sorbent mass may not affect separation and resolution of the peptides. Due to all these reasons, the C_{18} columns with maximum retention were studied further to determine if it was possible to elute interfering drugs at lower methanol concentrations retaining GnRH on the column for later elution with higher concentrations of methanol.

It was difficult to obtain good separation of analytes using non-polar SPE methods. Peak widths of analytes eluted are a function of molecular weight. The silica adsorbent surfaces are porous and the majority of the hydrophobic interactive surface is inside the pores where the analytes should enter to be adsorbed and separated. It is recommended that small analytes (<~2000 MW) could separate on maximum surface silica particles of 100A° (Geng & Regnier, 1984). The porosity of the columns we used was 55A°, but the minimal difference in non-polar (hydrophobic) character between the analytes (GnRH, MW 1212; methoxamine, MW 247; bicuculline, MW

509) could be one of the reasons that sharper peaks and better resolution was not obtained.

As we found little difference in hydrophobic interaction between the compounds, differences in ionic interactions were then investigated by comparing interactions with endcapped and non-endcapped sorbents. With C_{18} EC and NEC columns, GnRH eluted at 40 and 70% methanol, respectively. This was expected as EC columns have reduced number of surface silanol groups compared with NEC; therefore, the secondary ionic interactions of GnRH with the EC column were not as strong, leading to earlier elution. When methoxamine or bicuculline was applied to the columns, they started to elute at 30% in EC and at 50% methanol in NEC columns. This suggests that methoxamine and bicuculline have less secondary ionic interactions with the free silanol groups in the columns. Ionic interactions are in the range 50 to 200 kcal/mol and stronger than hydrophobic interactions which have binding energies ranging from 1 to 10 kcal/mol (Hennion, 1999). This difference in binding energies explains the important role of the residual silanol groups on silica even if there are a very small number of silanol groups. It also explains why desorption is not always straightforward, because one has to keep in mind that a strong interaction can be attractive for the extraction step, but then more difficult to break in the elution step.

Following the principle of SPE, if analyte (GnRH) elution occurs at a given percent organic eluent, it is recommended to use the percent just below to remove any interference (methoxamine or bicuculline) (Chloupek *et al.*, 1992). However, when this practice was followed by applying 30 and 50% methanol to the EC and NEC columns respectively, methoxamine or bicuculline was not eluted from mixture also containing GnRH. This suggests that the basic mechanism of eluting the compounds at lower concentrations, compared to those for GnRH, did not apply in the present conditions possibly due to modification by GnRH of the compound interaction with the columns and due to ionic interaction between GnRH and compounds. Using higher concentrations of methanol was the other option to elute the compounds, but this could have resulted in the elution of both GnRH and the compounds in the same fraction. When the analyte of interest elutes partially with matrix interference components during the elution step, it is advisable to increase gradually the concentration of the

organic eluent and decrease the eluent volume used (Chloupek *et al.*, 1992). To attempt to obtain good separation instead of using 1.5 ml methanol of higher concentration for the interference wash, we used 0.5 ml methanol of higher concentration per washing. The aim was to elute GnRH and compound into two different fractions of the same concentration of methanol, one with maximum compound and other with maximum GnRH, separated by at least one fraction with no GnRH-like activity. By applying 60 and 70% methanol to NEC columns and 50 and 55% methanol to EC columns, methoxamine and GnRH appeared to separate into two consecutive fractions, however, for unknown reasons this was not repeatable in subsequent attempts.

Next, we attempted to utilize the secondary ionic interactions of the analytes and the NEC silica columns. Highly energetic ionic interactions can occur when silanol groups are ionized and when analytes are positively charged. The *pK*a of a silanol group is not easy to determine because it depends on the exact experimental conditions. However, it is considered that at pH 2 it is uncharged and above 3 it becomes increasingly dissociated. The effect of the negative charge of the silanol groups can unambiguously observed above pH 4. On the other hand analytes (GnRH, methoxamine and bicuculline) contain amino groups or nitrogen and can be protonated depending on the pH. Even if the number of silanol groups is very low, these interactions are much stronger than hydrophobic ones and are difficult to disrupt (Hennion, 1999). We tested different pH and methanol concentration combinations. In the NEC columns, there was no convincing separation with 55 and 60% methanol at pH 3, however, in the EC columns 45% methanol at pH 3 separated GnRH and methoxamine into two non-consecutive fractions with low GnRH-like activity in the intervening fraction. Unfortunately, when this experiment was repeated, the drugs appeared to be eluted earlier than GnRH but the fractions were consecutive. Similarly, with methanol pH 3.0 and concentration 40%, bicuculline appeared to be eluted one fraction early in EC columns but complete separation was not achieved. These findings suggested that protonation of these basic compounds with the use of NEC columns had detrimental effects on separation due to interactions of these compounds

with the more residual silanol groups on silica-based stationary phases in NEC columns (Devi *et al.*, 1988; Hubert *et al.*, 1994).

Ultimately, we reached the conclusion that 40 to 45% methanol (pH 2.5-3.0) in C_{18} sorbent mass could be used to onlt partially separate the methoxamine and bicuculline from the GnRH and was not suitable for complete separation. SPE method could not be validated with respect to precision and reproducibility and that SPE-HPLC combination may be more appropriate if these compounds were to be reliable separated from GnRH to prevent interference with radioimmunoassays. The alternative option of using LH release by pituitary tissue as a bio-assay for GnRH was followed in subsequent chapters of this thesis.

(A)

NON-ENDCAPPED

	Methanol (%)	0.5ml each % of Methanol						100% Methanol (0.5ml each)		
		I	2	3	4	5	6	1	2	3
GnRH (pg/ml)	60	0	0	0	0	0	2	16	10	3
GnRH+Methoxamine (pg/ml)	60	0	0	1	3	1	25	18	2	2
GnRH	70	0	0	5	13	12	7	0	0	2
GnRH+Methoxamine	70		1	9	21	7	3	4	1	2
GnRH	80	1	9	16	2	1	3	0	1	1
GnRH+Methoxamine	80	2	15	15	4	2	2	0	0	1
GnRH	90	3	31	8	2	2	0	0	0	2
GnRH+Methoxamine	90	4	34	14	7	2	0	0	0	1
GnRH	100	6	28	9	3	6	0	0	1	3
GnRH+Methoxamine	100	4	24	25	6	0	0	0	1	3

ENDCAPPED

	Methanol (%)	0.5ml each % of Methanol							100% Methanol (0.5ml each)		
		I	2	3	4	5	6	7	1	2	3
GnRH (pg/ml)	35	1	0	0	1	11	9	6	6	2	2
GnRH+Methoxamine (pg/ml)	35	1	0	1	2	2	2	6	15	6	1
GnRH	40	1	1	0	2	20	8	3	0	1	1
GnRH+Methoxamine	40	2	1	1	1	0	12	13	4	2	1
GnRH	45	1	0	1	22	7	2	0	0	0	1
GnRH+Methoxamine	45	1	1	3	3	12	18	2	0	1	1
GnRH	50	1	4	2	37	0	1	0	0	0	1
GnRH+Methoxamine	50	1	4	10	23	14	6	0	0	2	1
GnRH	55	3	0	18	23	1	0	0	0	1	0
GnRH+Methoxamine	55	1	18	30	7	1	0	0	0	1	0

(B)

NON-ENDCAPPED

	Methanol (%)	0.5ml each % of Methanol										100% (0.5ml)
		1	2	3	4	5	6	7	8	9	10	1
GnRH (pg/ml)	50	1	0	0	0	0	1	0	13	4	0	0
GnRH+Methoxamine (pg/ml)	50	1	0	0	0	0	1	4	16	8	3	0
GnRH	55	1	0	0	0	0	1	0	1	10	2	0
GnRH+Methoxamine	55	1	0	0	0	0	1	22	8	1	0	0
GnRH	60	0	0	0	0	0	17	3	8	3	0	0
GnRH+Methoxamine	60	0	0	0	0	10	17	4	0	0	0	0
GnRH	65	1	0	0	0	6	10	2	0	0	0	0
GnRH+Methoxamine	65	1	0	0	3	19	7	3	0	0	0	0

ENDCAPPED

	Methanol (%)	0.5ml each % of Methanol						100% (0.5ml)
		1	2	3	4	5	6	1
GnRH (pg/ml)	55	0	0	0	24	0	0	0
GnRH+Methoxamine (pg/ml)	55	0	0	1	35	3	0	0
GnRH	60	0	0	0	18	0	0	0
GnRH+Methoxamine	60	0	0	3	39	0	0	0

Table 6.1 Elution of GnRH or methoxamine from 500mg sorbent mass C_{18} columns (endcapped; EC or non-endcapped; NEC). Samples of GnRH or GnRH + methoxamine were applied to separate EC or NEC columns and eluted with various concentrations of methanol (pH 5.0). Each column was washed with 0.5ml of respective concentration of methanol, repeated 6-10 times. Final wash was carried out with 100% methanol. Fractions were collected and GnRH or GnRH-like activity (pg/ml) of methoxamine was determined by radioimmunoassay. A) Values in red indicate the fraction numbers where GnRH started to elute in GnRH only columns. Values in blue indicate the elution of methoxamine or GnRH in mixed columns. Circled areas indicate the fractions where methoxamine may have eluted earlier compared to GnRH only columns. B) Results from another similar experiment revealing lack of reproducibility.

(A)

NON-ENDCAPPED

	Methanol		0.5ml each % of Methanol										100% (0.5ml)
	pH	%	I	2	3	4	5	6	7	8	9	10	1
GnRH (pg/ml)	3	55	1	1	0	0	0	25	4	1	2	2	4
GnRH+Methoxamine (pg/ml)	3	55	1	12	3	4	6	6	0	0	0	1	2
GnRH	3	60	1	12	20	3	1	1	0	0	0	0	0
GnRH+Methoxamine	3	60	0	35	12	4	0	0	0	0	0	1	1
GnRH	11	55	0	1	1	2	0	0	0	0	1	2	20
GnRH+Methoxamine	11	55	1	1	2	1	1	0	2	3	6	7	20
GnRH	11	60	1	0	2	0	1	2	3	6	4	10	7
GnRH+Methoxamine	11	60	1	1	1	1	2	1	7	7	8	7	12

ENDCAPPED

	Methanol		0.5ml each % of Methanol									100% (0.5ml)
	pH	%	I	2	3	4	5	6	7	8	9	1
GnRH (pg/ml)	3	45	3	0	16	3	2	2	0	0	0	0
GnRH+Methoxamine (pg/ml)	3	45	3	8	3	12	7	3	2	0	0	0
GnRH	3	50	2	6	11	3	2	0	1	0	0	0
GnRH+Methoxamine	3	50	2	15	8	8	3	1	1	1	0	0
GnRH	11	45	0	3	0	0	0	0	0	1	0	6
GnRH+Methoxamine	11	45	0	2	7	0	0	0	0	0	4	52
GnRH	11	50	0	0	0	0	0	0	0	0	0	0
GnRH+Methoxamine	11	50	0	0	0	0	0	0	0	0	1	3

(B)

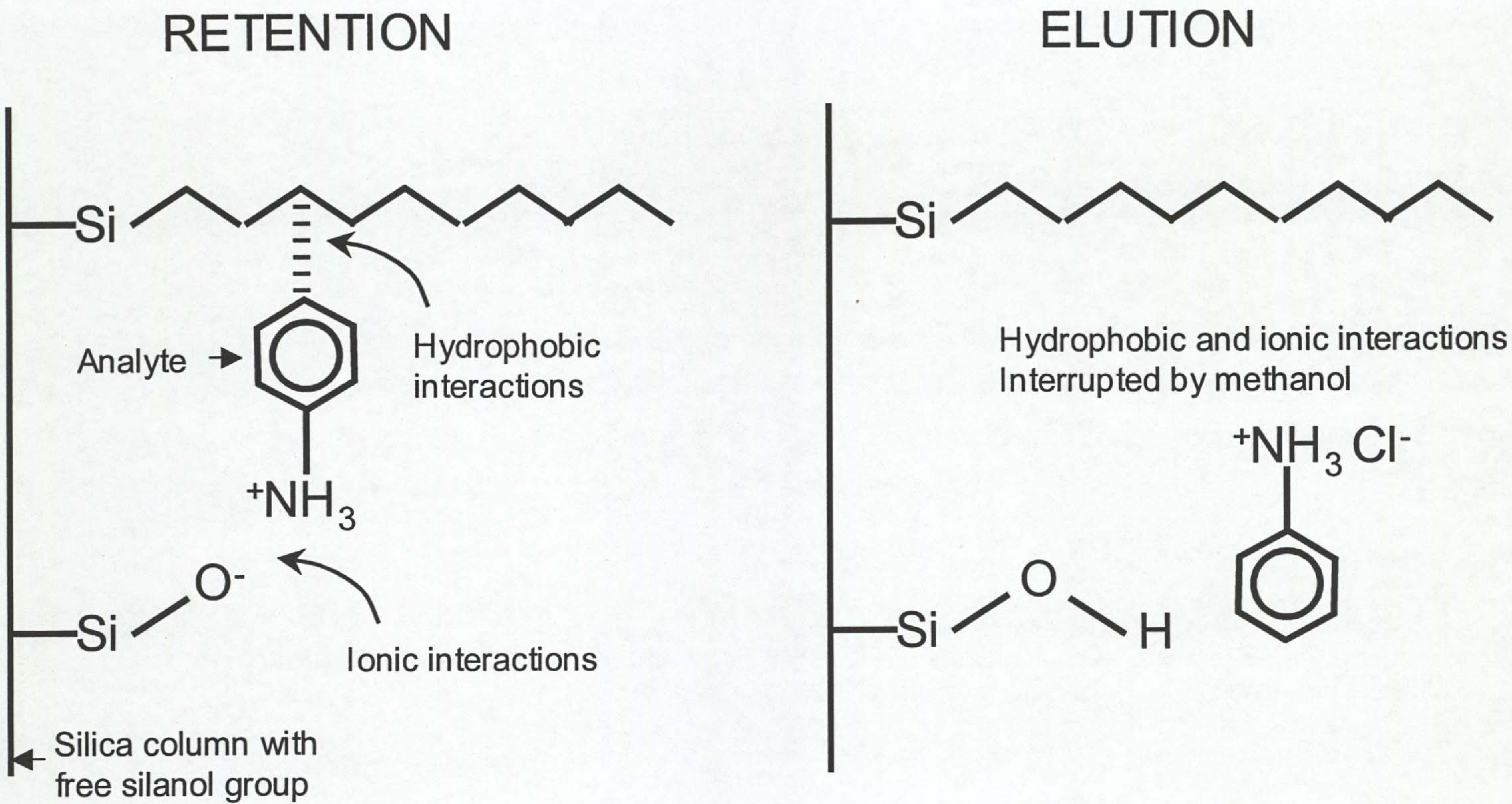
ENDCAPPED

	Methanol		0.5 ml each % of Methanol					
	pH	%	1	2	3	4	5	6
GnRH (pg/ml)	2.5	35	2	0	11	10	6	1
GnRH+Methoxamine (pg/ml)	2.5	35	2	18	11	7	3	2
GnRH	2.5	40	1	2	7	6	3	5
GnRH+Methoxamine	2.5	40	2	7	7	6	2	2
GnRH	2.5	45	1	2	7	6	2	8
GnRH+Methoxamine	2.5	45	1	11	7	6	2	3
GnRH	5.0	40	2	2	10	12	4	8
GnRH+Methoxamine	5.0	40	2	6	2	1	0	40
GnRH	5.0	45	1	2	4	10	5	12
GnRH+Methoxamine	5.0	45	1	8	2	1	0	37

Table 6.2 Elution of GnRH or methoxamine from 500mg sorbent mass C_{18} columns (endcapped; EC or non-endcapped; NEC). Samples of GnRH or GnRH + methoxamine were applied to separate EC or NEC columns and eluted with various combinations of pH and methanol concentration. Each column was washed with 0.5ml of respective concentration of methanol, repeated 6-10 times. Final wash was carried out with 100% methanol. Fractions were collected and GnRH or GnRH-like activity (pg/ml) of methoxamine was determined by radioimmunoassay. Values in red indicate the fraction number where GnRH started to elute in GnRH only columns. Values in blue indicate the elution of methoxamine or GnRH in mixed columns. Circled areas in A) and B) indicate the fractions where methoxamine may have eluted earlier compared to GnRH only columns.

ENDCAPPED									
	Methanol		0.5 ml each % of Methanol						
	pH	%	1	2	3	4	5	6	7
GnRH (pg/ml)	3	35	0	4	5	2	1	3	5
GnRH+Bicuculline (pg/ml)	3	35	0	13	4	2	1	8	5
GnRH	3	40	0	5	26	5	7	1	2
GnRH+Bicuculline	3	40	2	22	10	3	4	0	0
GnRH	3	45	0	16	3	4	0	0	0
GnRH+Bicuculline	3	45	0	19	10	2	0	10	4
GnRH	4	35	0	11	17	5	7	0	0
GnRH+Bicuculline	4	35	1	24	4	0	0	0	0
GnRH	4	40	1	25	2	6	4	0	0
GnRH+Bicuculline	4	40	1	42	4	4	0	0	0
GnRH	4	45	0	39	23	11	3	0	0
GnRH+Bicuculline	4	45	2	44	5	5	0	0	4

Table 6.3 Elution of GnRH or bicuculline from 500mg sorbent mass C_{18} columns (endcapped; EC). Samples of GnRH or GnRH + bicuculline were applied to separate EC columns and eluted with various combinations of pH and methanol concentration. Each column was washed with 0.5ml of respective concentration of methanol, repeated 7 times. Fractions were collected and GnRH or GnRH-like activity (pg/ml) of bicuculline was determined in each fraction by radioimmunoassay. Values in red indicate the fraction number where GnRH started to elute in GnRH only columns. Values in blue indicate the elution of bicuculline or GnRH in mixed columns. Circled areas indicate fractions where bicuculline may have eluted earlier compared to GnRH only columns.



RETENTION: Primarily due to hydrophobic interactions though ionic mechanisms also exist.
ELUTION: Hydrophobic as well as ionic interactions can be disrupted with an organic eluent (methanol) which solvates the surface of the sorbent as well as the analyte.

Fig 6.1 Principle retention and elution mechanisms of non-polar sorbents.

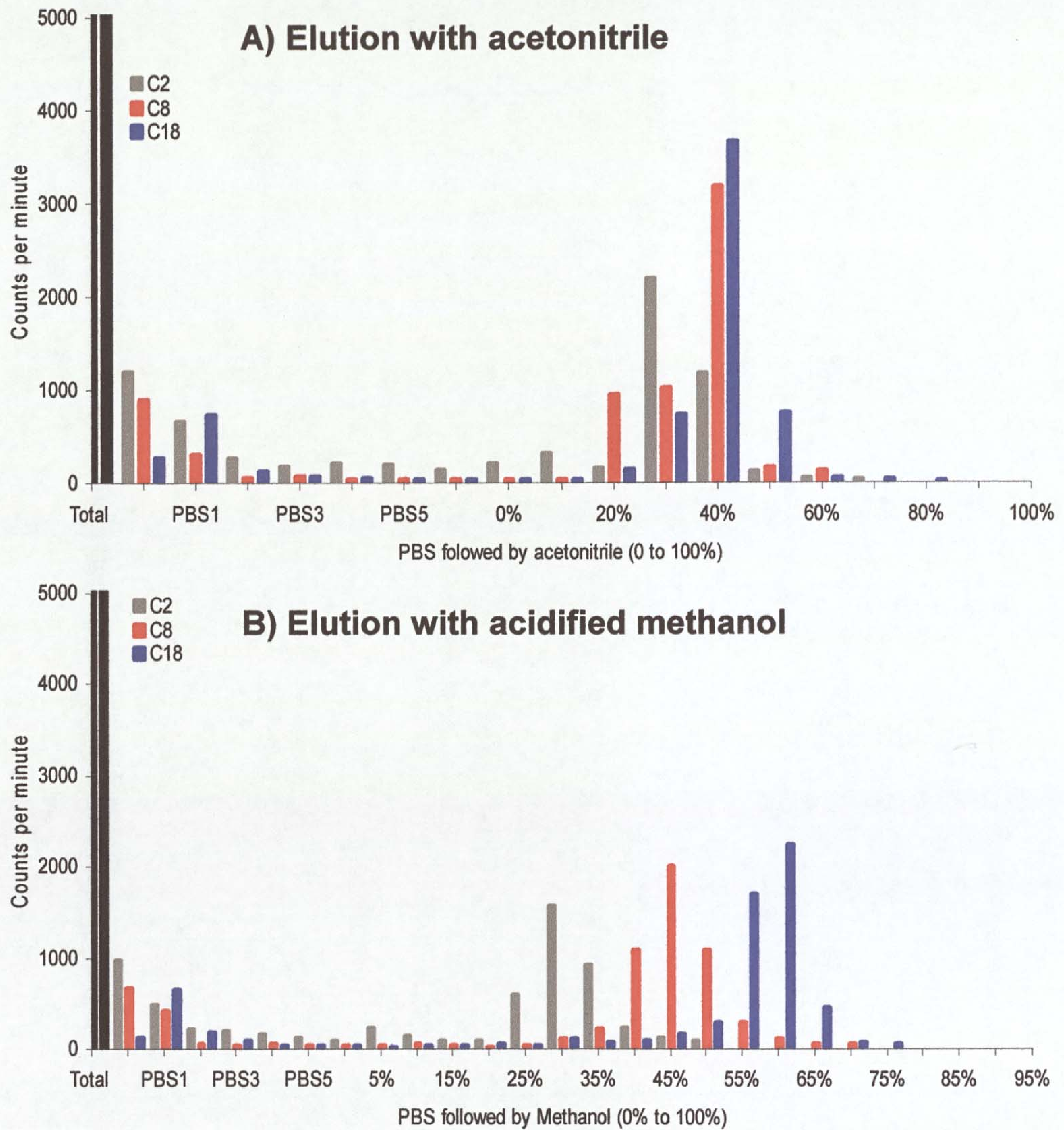
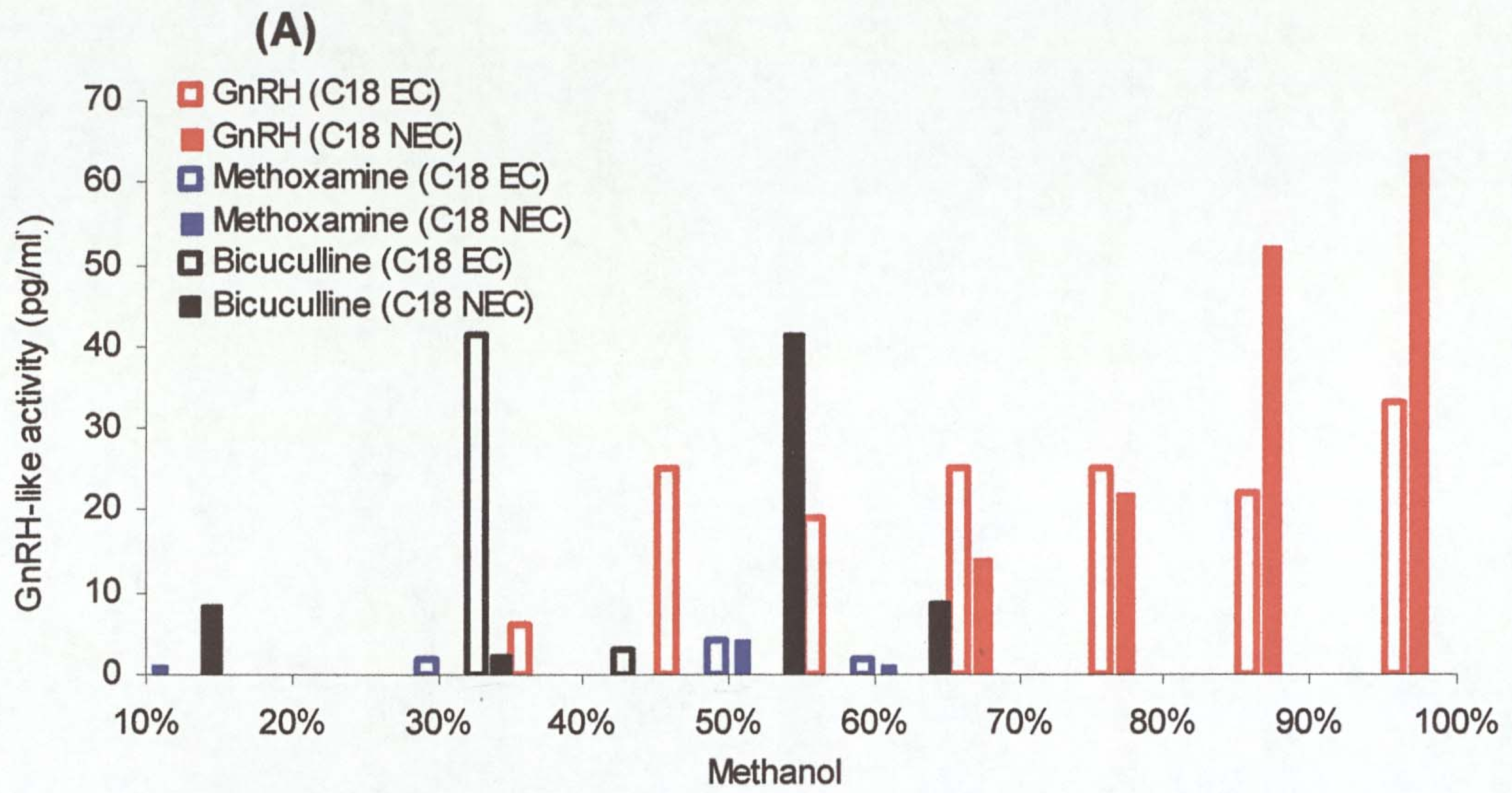


Fig 6.2 Elution of I^{125}GnRH from 100mg sorbent mass C_2, C_8 and C_{18} columns (endcapped). Approximately 5000cpm I^{125}GnRH applied to each column, washed with 0.1M phosphate buffer saline (PBS, pH 7.4, 2ml six times), followed by elution with increasing concentrations of A) acetonitrile or B) acidified methanol (0 to 100%, in 10% steps one wash of 1.5ml each).



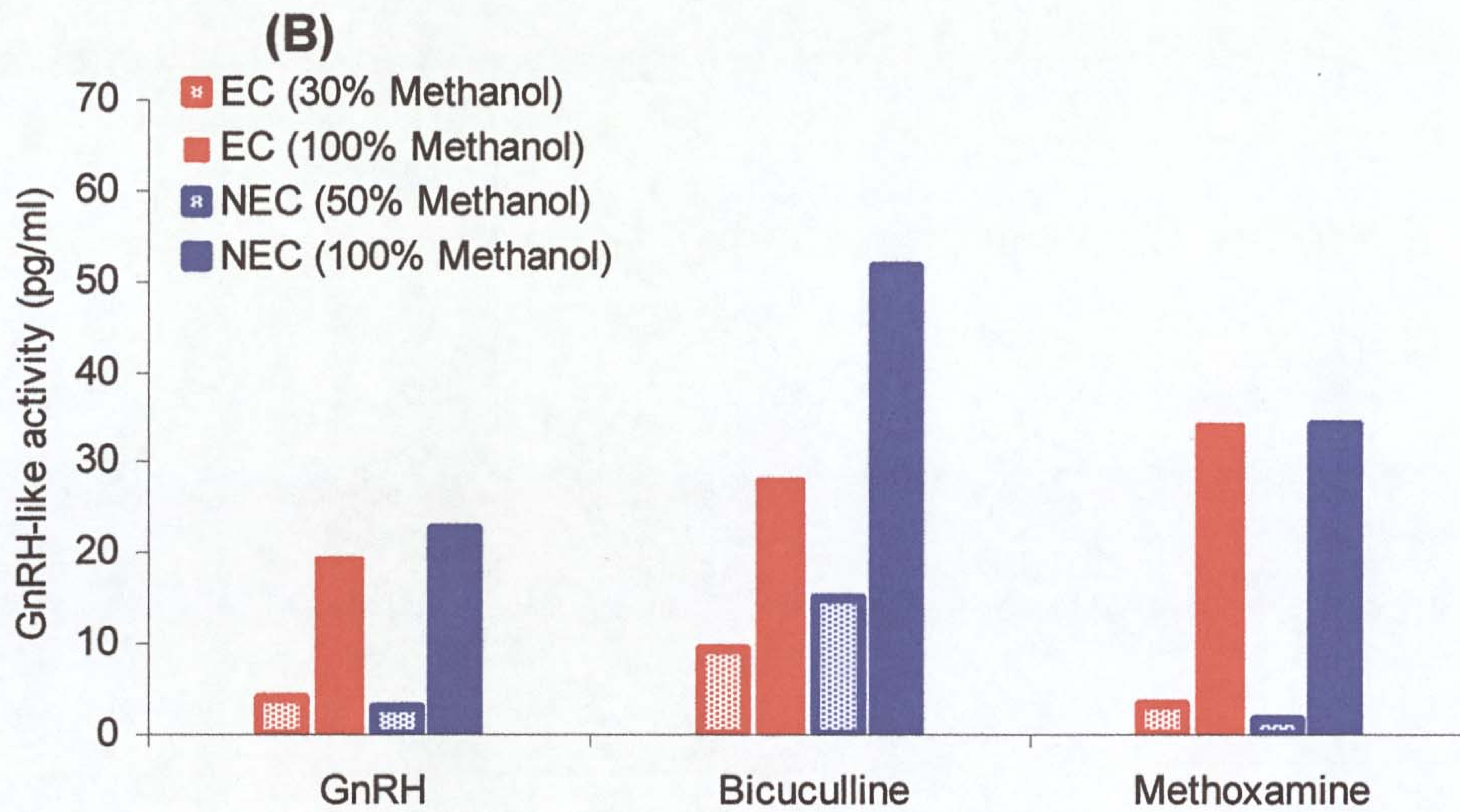


Fig 6.3 Elution of GnRH, methoxamine or bicuculline from 500mg sorbent mass C_{18} columns (endcapped; EC or non-endcapped; NEC). Samples were applied to separate columns, washed with 0.1M phosphate buffer saline (2ml six times, pH 7.4), followed by elution with 1.5ml of A) increasing concentrations (0-100%) of acidified methanol or B) 30 (EC) or 50% (NEC) acidified methanol followed by 100% acidified methanol. Fractions were collected and GnRH-like activity (pg/ml) of methoxamine or bicuculline determined by radioimmunoassay.

Chapter 7

GABA control of GnRH release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

GABA control of GnRH release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

Abstract

The present study aims to examine the influence of $GABA_{A \text{ or } B}$ receptors on GnRH release *in vitro* and determine whether E_2 modulates GABA-GnRH interaction. Ten minutes after ewe sacrifice, saggital midline hypothalamic slices (from the anterior preoptic area to mediobasal hypothalamus with median eminence, 2 mm thick, 2 per sheep) were dissected, placed in oxygenated MEM-α at 4°C and within the 2 h were singly perifused at 37°C with oxygenated MEM-α (pH 7.4; flow rate 0.15 ml/min), either with or without E_2 (24 pg/ml). After 4 h equilibration, 10 min fractions were collected for 4 h interposed with a 10 min exposure at 60 min to specific $GABA_{A \text{ or } B}$ receptor agonist or antagonist at various doses (0.1-10 mM). The GABA receptor compounds interacted with the GnRH assay. Therefore, the net effect of all the GABA receptor compounds on immunoreactive GnRH release was obtained after subtracting the artefactual increase from appropriate control chambers. The $GABA_A$ agonist (muscimol 10 mM; No E_2, n=2, With E_2, n=11) did not influence net immunoreactive GnRH concentrations, however $GABA_B$ agonist stimulated ($p<0.05$) net immunoreactive GnRH release at 90 min (baclofen 10 mM; No E_2, n=5: 0.5±1.2 to 3.9±1.0, With E_2, n=10: 3.1±1.3 to 6.2±1.3 pg/ml). Furthermore, net immunoreactive GnRH release increased ($p<0.05$) at 100-130 min after exposure to 10 mM $GABA_{A \text{ or } B}$ antagonists (bicuculline, No E_2, n=2, With E_2, n=7; CGP52432, No E_2, n=2, With E_2, n=11). In the absence of E_2, the $GABA_A$ antagonist stimulated net immunoreactive GnRH release was greater ($p<0.05$) than in the presence of E_2.

The subsequent bioactivity of released GnRH on anterior pituitary tissue (collected at the same time) was also studied in a sequential double-chamber perifusion system. After exposure of hypothalamic slices to $GABA_A$ antagonist, the released GnRH stimulated net LH release from pituitary fragments ($p<0.05$; n=11, -0.8±7.6 to 41.2±23.1 ng/ml). This confirms that the $GABA_A$ antagonist stimulated GnRH release that was biologically active. At the end of the collection period,

hypothalamic slices or anterior pituitary fragments responded to KCl (100 mM) with GnRH or net LH efflux ($p<0.05$). In summary, GnRH release from the hypothalamus is predominantly under $GABA_A$ receptor inhibitory control and this effect is attenuated in the presence of E_2.

Introduction

The negative correlation between luteinising hormone (LH) and γ-aminobutyric acid (GABA) release suggests that GABA neurones exert an inhibitory influence on gonadotrophin releasing hormone (GnRH) neurones (Robinson, 1995). In the ewe, GABA terminals are in close contact with GnRH neurones in the preoptic area (POA) (Jansen *et al.*, 2003). In other species, it has been shown that GABA terminals synapse directly on GnRH cell bodies and $GABA_A$ receptors colocalise with GnRH neurones in the POA (Petersen *et al.*, 1993; Jung *et al.*, 1998; Spergel *et al.*, 1999; Sim *et al.*, 2000). In the mediobasal hypothalamus (MBH) and in the external layer of the median eminence (ME), there are also high affinity uptake sites for GABA in addition to the presence of GABA neurones and GABA receptor mRNA (Tappaz *et al.*, 1980; Anderson & Mitchell, 1986a; Horvath *et al.*, 1997; Ovesjo *et al.*, 2001). All this evidence suggests that GABA may act within the POA as well as in the ME to control GnRH release. Furthermore, there is controversy over the involvement of the different receptor subtypes for GABA control of GnRH release in sheep. Some propose that $GABA_A$ receptors are important while other advocate $GABA_B$ receptor-mediated control of GnRH release (Scott & Clarke, 1993a; Scott & Clarke, 1993b; Ferreira *et al.*, 1996; Ferreira *et al.*, 1998).

Activation of oestradiol receptors (ERα) on GABA neurones could mediate E_2-negative feedback on GnRH neurones as E_2 treatment increases GABA concentrations in the POA of ovariectomized (OVX) ewes (Robinson *et al.*, 1991; Herbison *et al.*, 1993b). The other principal change in GABA metabolism is the later marked decrease in GABA tone just before the generation of the E_2-induced preovulatory LH surge in the ewe (Robinson *et al.*, 1991; Robinson & Kendrick, 1992a). Glutamic acid decarboxylase (GAD, a biosynthetic enzyme for GABA) expression in the POA also

decreases before the onset of an endogenous or E_2-induced LH surge (Morello *et al.*, 1989; Herbison & Dyer, 1991; Herbison *et al.*, 1992; Unda *et al.*, 1995). Whether E_2 influences GABA receptor subtype-mediated functions to control different pattern of GnRH release is not clear in ewe.

Overall, it appears that GABA may act at multiple sites to affect GnRH-LH release. Therefore, in the present study we investigated the effect of the GABA system in regulation of GnRH release within ewe hypothalamus, using an *in vitro* perifusion model. Secondly, we aimed to determine whether effect of GABA system depends on E_2 background. An *in vitro* approach was essential to avoid any confounding effects of GABA receptor compounds or E_2 on other body systems. We extended the perifusion system by exposing anterior pituitary fragments to hypothalamic effluent to confirm that the GnRH released was biologically active.

Materials and Methods

Hypothalamic slice and/or anterior pituitary tissue perifusion in vitro

The perifusion was performed as described in detail elsewhere (chapter 2). After a 4 h equilibration period, medium effluent fractions were collected for 4 h.

Treatments

Hypothalamic slices were perifused alone with media, with or without E_2 (24 pg/ml, Sigma-Aldrich). After a 60 min collection period, slices were treated for 10 min with vehicle (MEM-α, with or without E_2, n=10 perifusion chambers each), or different doses of a $GABA_A$ receptor agonist (muscimol, Sigma-Aldrich; without E_2, 0.1 or 1 mM, n=4 each; 10 mM, n=2; with E_2, 0.1 or 1 mM, n=4 each; 10 mM, n=11) or antagonist (bicuculline, Sigma-Aldrich; without E_2, 0.1 mM, n=2; 1 mM, n=8; 10 mM, n=2; with E_2, 0.1 mM, n=4; 1 mM, n=5; 10 mM, n=7); or a $GABA_B$ receptor agonist (baclofen, Sigma-Aldrich; without E_2, 0.1 mM, n=4; 1 or 10 mM, n=5 each; with E_2, 0.1 mM, n=4; 1 mM, n=5; 10 mM, n=10) or antagonist (CGP52432, Tocris Cookson ltd; without E_2, 0.1 mM, n=4; 1 mM, n=5; 10 mM, n=2; with E_2, 0.1 or 1 mM, n=5; 10 mM, n=10).

Anterior pituitary fragments alone (vehicle, n=7; muscimol 10 mM, n=5; bicuculline 10 mM, n=6; baclofen 10 mM, n=6; CGP52432 10 mM, n=6) or in series with the hypothalamic slices (vehicle, n=7; muscimol 10 mM, n=9; bicuculline 10 mM, n=11; baclofen 10 mM, n=9; CGP52432 10 mM, n=8) were perifused without E_2. At the end of the perifusion, all slices or anterior pituitary fragments were exposed to a depolarizing stimulus of KCl (100 mM) for 10 min after 180 min of perifusate collection.

GnRH assay

GnRH was measured by radioimmunoassay (chapter 2). Sensitivity of the assay was 5.2 pg/ml. Inter- and intra-assay coefficients of variation were 7.7% and 5.8%, respectively. There was no interaction in the GnRH assay with E_2 or KCl. However, bicuculline and CGP52432 interacted with the GnRH assay. Therefore, empty chambers were run with media perifusate collected during the equilibration period. This part of experiment was performed in a manner identical to that of the hypothalamic slice perifusions alone.

LH assay

LH was measured using radioimmunoassay (chapter 2). Sensitivity of the assay was 0.3 ng/ml. Inter- and intra-assay coefficients of variation were 7.5% and 8.2%, respectively. There was no interaction in the LH assay with GABA receptor compounds or KCl at the concentrations used in this study.

Statistical analysis

Basal release, with or without E_2, was defined as the mean immunoreactive GnRH release in perifusate fractions collected for 60 min, prior to start of GABA agonist or antagonist treatment. For dose response studies (Figs 7.1A-D, 7.2A-D), pre- or post-treatment values were calculated by averaging the total immunoreactive GnRH concentrations of individual chambers at 60 and 70 min for pre-treatment, and at 80 and 90 min for post-treatment. Two time points after treatments were chosen as the mean time of peak response was variable between chambers. Similarly, pre- and post-

KCl (Figs 7.1E-H, 7.2E-H) concentrations of total immunoreactive GnRH were calculated by averaging 180 and 190, or 200 and 210 min fractions, respectively. The net immunoreactive GnRH release (Figs 7.3C-D, 7.4C-D, 60-150 min) was calculated by subtracting the immunoreactive GnRH values of empty chamber perifusates (because of GABA receptor compound interaction in the GnRH assay) from total immunoreactive GnRH values of hypothalamic slice-containing chamber perifusates (Figs 7.3A-B, 7.4A-B). In the perifusion experiments of anterior pituitary fragments alone, LH was released after exposure to GABA receptor compounds or KCl. Thus, the net LH release (Fig 7.5C, 7.6C), reflecting the effect of net bioactive GnRH released from hypothalamic slices, was calculated by subtracting the mean LH of pituitary-containing chambers from in series pituitary chambers in respective groups (Figs 7.5A-B, 7.6A-B).

Statistical analysis was performed as described previously (chapter 4)

Results

The mean basal (10-60 min) immunoreactive GnRH released by hypothalamic slices perifused with medium containing E_2 was higher ($p<0.05$, 14.5±0.1 pg/ml, n=48, Figs 7.3B, 7.4B) than slices perifused with medium containing no E_2 (12.6±0.1 pg/ml, n=21, Figs 7.3A, 7.4A).

The effects of various concentrations (0.1-10 mM) of $GABA_{A\ or\ B}$ agonists or antagonists on the release of immunoreactive GnRH from the perifusion containing hypothalamic slices alone are shown in Figs 7.1 and 7.2. Exposure of hypothalamic slices to 10 mM $GABA_{A\ or\ B}$ antagonists stimulated GnRH release (Figs 7.1D, 7.2D). The release of GnRH from hypothalamic slices during the post-treatment period (80-150 min) following exposure to 10 mM $GABA_{A\ or\ B}$ agonists or antagonists is emphasized in Figs 7.3 and 7.4 (A, B).

Any possible interactions of 10 mM $GABA_{A\ or\ B}$ agonists or antagonists with the GnRH assay, are shown in Figs 7.3 and 7.4 (A, B; compound without slice). Unfortunately, exposure to 10 mM $GABA_{A\ or\ B}$ antagonists resulted in an increase in immunoreactive GnRH in chambers with or without hypothalamic slices present (Figs

7.3A-B, 7.4A-B). However, subtracting results obtained with no slice from results with slices, the increase in net immunoreactive GnRH is represented in Figs 7.3 and 7.4 (C, D).

The net immunoreactive GnRH induced by 10 mM $GABA_{A\ or\ B}$ antagonists or $GABA_B$ agonist occurred between 90-130 min, both in the slices perifused with (Figs 7.3D, 7.4D) or without E_2 (Figs 7.3C, 7.4C). The increase in net GnRH induced by 10 mM $GABA_A$ antagonist was greater than that after vehicle alone (Figs 7.3C-D). Furthermore, there was more net GnRH released at 100 min after $GABA_A$ antagonist exposure in the absence of E_2 than in the presence of E_2 (Figs 7.3C-D).

Figs 7.5 and 7.6 (A, B) show the effect of 10 mM $GABA_{A\ or\ B}$ agonists or antagonists on LH release from anterior pituitary tissue alone, or from the anterior pituitary tissue containing chambers placed in series with hypothalamic slices. Application of 10 mM $GABA_{A\ or\ B}$ antagonists or $GABA_B$ agonist stimulated LH release in both situations, i.e. pituitary alone or hypothalamus and pituitary placed in series (Figs 7.5B and 7.6A-B). The increase in net LH released from the pituitary due to the presence of net bioactive GnRH in hypothalamic perifusate is represented in Figs 7.5C and 7.6C. After exposure to 10 mM $GABA_A$ antagonist, GnRH released in the hypothalamic perifusate had stimulatory effect on net LH released from the pituitary tissue (Fig 7.5C). However, there was no change in net LH with 10 mM $GABA_B$ agonist or antagonist (Fig 7.6C).

At the end of each experiment, potassium chloride exposure stimulated an increase in immunoreactive/bioactive GnRH or LH confirming that both hypothalamic slices and anterior pituitary tissue were viable during the experimental conditions (Figs 7.1 and 7.2 E-H, Figs 7.3 to 7.6 A-B).

Discussion

Basal release of GnRH from the hypothalamic slices was augmented by the presence of E_2 in the perifusion media. These results confirm our previous *in vitro* data (chapter 3) and are in agreement with *in vivo* studies in the ewe reporting an increase

in GnRH concentrations in portal blood when E_2 concentrations are high (Moenter *et al.*, 1990).

The results of the present study demonstrate that inhibitory control of GABA on GnRH system within the hypothalamus is predominantly through $GABA_A$ receptors with attenuation of this inhibition in the presence of E_2. The increase in net LH release from pituitary tissue in response to $GABA_A$ antagonist further confirms that 1) a $GABA_A$ antagonist stimulates net immunoreactive/bioactive GnRH release from the hypothalamus, 2) total immunoreactive GnRH measured by radioimmunoassay contains bioactive GnRH released from hypothalamus and is not merely the detection of an artefactual increase in immunoreactive GnRH values due to GABA receptor compound interaction with the GnRH assay.

The blockade of $GABA_A$ receptors enhances GnRH release from ewe hypothalamic slices *in vitro* and this is extended to biological activity on anterior pituitary tissue. This is consistent with previous studies in the sheep showing an GABA inhibitory effect mediated through $GABA_A$ receptors both in the POA and in the MBH (Scott & Clarke, 1993b; Ferreira *et al.*, 1996; Tomaszewska-Zaremba *et al.*, 2003). This influence of $GABA_A$ receptor antagonists might operate through removal of the $GABA_A$ receptor-induced hyperpolarisation of GnRH neurones (DeLorey & Olsen, 1992). Alternatively, $GABA_A$ receptors may interact with excitatory neurones impinging on GnRH neurones (Thind & Goldsmith, 1995; Bourguignon *et al.*, 1997). The removal of GABA inhibition of excitatory neurones would increase excitatory input, causing an increase in GnRH.

It is worth noting that the effect of the $GABA_A$ antagonist on net GnRH release was markedly higher than the $GABA_B$ receptor compounds (Figs 7.3C-D and 7.4C-D). Furthermore, $GABA_B$ receptor-induced net GnRH release from the hypothalamus (Figs 7.4C-D) was not sufficient to stimulate net LH from the pituitary tissue (Figs 7.6C). Both these observations reveal a predominant $GABA_A$, but not $GABA_B$, receptor-mediated control of GnRH neurones. This could not be simply due to the more effective dose of $GABA_A$ antagonist compared to $GABA_B$ antagonist because CGP52432, the $GABA_B$ antagonist, is among the most potent available $GABA_B$ antagonist with an IC-50 of 85 nM (Teoh *et al.*, 1996). In support, the present study

was done in the breeding season and the inhibition of LH secretion in ewes during the breeding season has been shown *in vivo* to be strongly controlled by $GABA_A$ receptors (Scott & Clarke, 1993a; Scott & Clarke, 1993b).

In the present study, high E_2 concentrations attenuate the inhibitory $GABA_A$ receptor control of net immunoreactive GnRH release (Figs 7.3C-D). These results are in agreement with the suggestion that high E_2 eventually decreases GABA turnover which in turn facilitates activation of GnRH neurones (Wagner *et al.*, 2001). In OVX ewes, there is a marked decrease in GABA tone just before generation of the GnRH/LH surge coincident with high E_2 concentrations (Robinson *et al.*, 1991). Furthermore, high E_2 concentrations may have altered the functions of α_1-adrenoreceptors present on GABA neurones (Han *et al.*, 2002). *In vivo* studies in rats and ewe demonstrate that with low E_2 concentrations, noradrenaline stimulates GABA to ultimately suppress GnRH release, but with high E_2 concentrations in the preovulatory period, noradrenaline release fails to activate GABA neurones. The precise reason for this uncoupling of the noradrenaline stimulatory influence upon GABA neurones prior to the LH surge is not clear (Herbison *et al.*, 1990; Robinson *et al.*, 1991; Herbison, 1997a). Nevertheless, decreased GABA turnover and/or reduced activation of GABA neurones by α_1-adrenoreceptors in a high E_2 milieu will ultimately lift the tonic inhibition of GnRH neurones by GABA, leading to the observed lower response to $GABA_A$ antagonist (Figs 3C-D; Fig 7.7).

Responses to $GABA_B$ compounds do not vary with the presence of E_2 suggesting that $GABA_B$ receptors are not involved in E_2 modulations of GnRH release. The control of GnRH release by E_2 via $GABA_B$ receptors was initially reported in anoestrous ewes (Scott & Clarke, 1993b). Furthermore, $GABA_B$ receptors are not thought to be related with GnRH/LH surge mechanisms as infusion of a $GABA_B$ antagonist had no effect on the expression of E_2-induced surges in OVX ewes (Jackson & Kuehl, 2004).

In the present study, we have also observed that GABA compounds can directly influence LH release from ewe anterior pituitary fragments. In the rat, anterior pituitary LH cells contain $GABA_{A \text{ and } B}$ receptor subunits (Mayerhofer *et al.*, 2001). Evidence for the direct GABA action on LH release from the anterior pituitary has

been provided by *in vitro* incubations of GABA receptor compounds with rat pituitary cells (Anderson & Mitchell, 1986b; Brann *et al.*, 1992). It was not the original intent of this study to investigate the effect of GABA receptor compounds on LH release from pituitary, although the greater release of LH from pituitaries perifused in series with hypothalamus compared to pituitaries alone further confirms that GABA receptor compounds stimulate net bioactive GnRH from the hypothalamus.

In conclusion, the present study emphasizes the role of $GABA_A$ receptors in controlling GnRH release within the hypothalamus. These results are the first to demonstrate *in vitro* that $GABA_A$ receptor-mediated control of GnRH release is influenced by E_2 in the ewe. Further investigation is needed to determine the precise mechanism(s) of E_2 influence on GABA interaction with other interneurones which ultimately regulate GnRH release.

$GABA_A$ receptor compounds

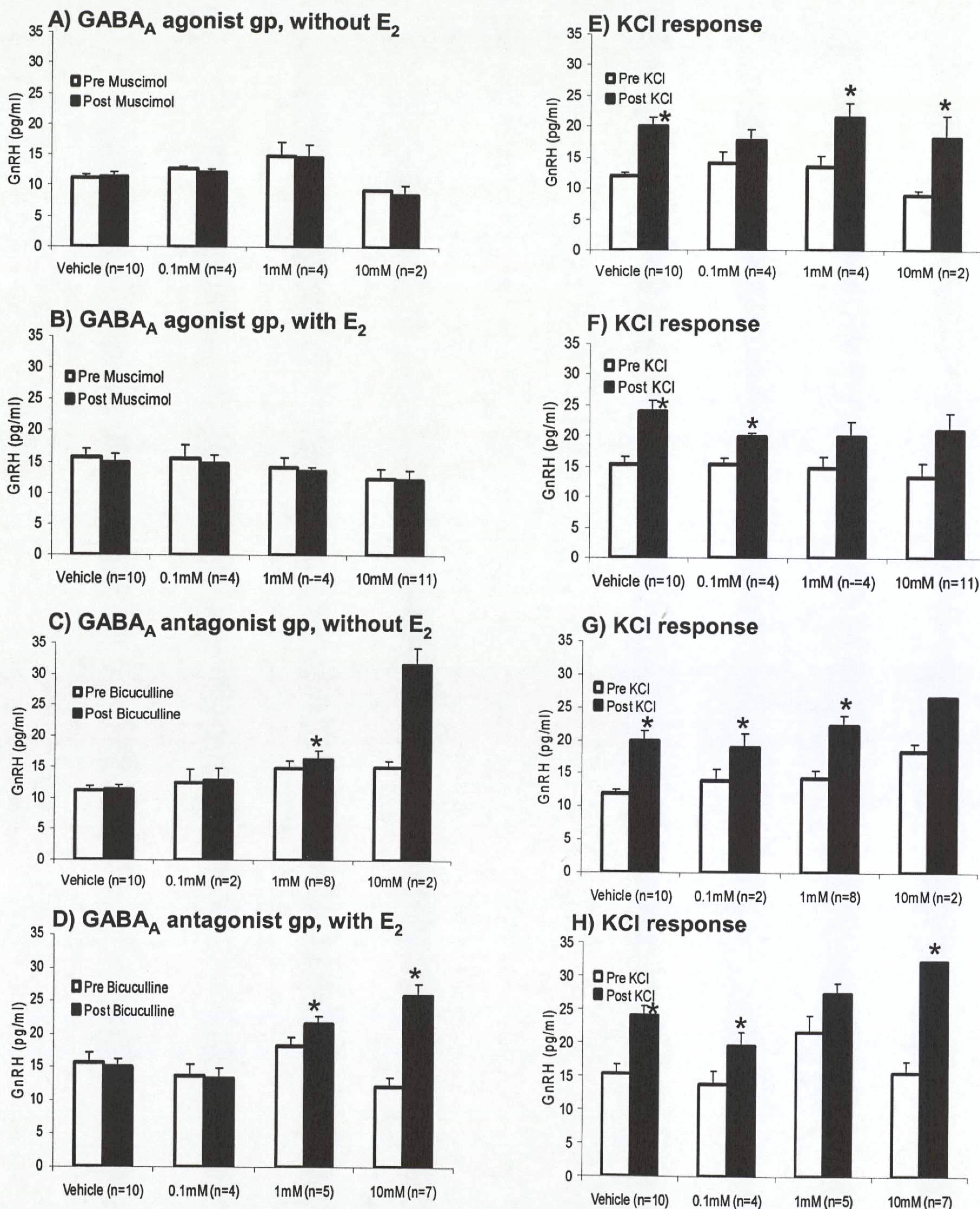


Fig 7.1 Effects of various doses (0.1mM-10mM) of $GABA_A$ agonist (muscimol) or antagonist (bicuculline) and KCl (100mM) challenge upon the release of total immunoreactive GnRH (mean±SEM) from ewe hypothalamic slices perifused with media alone or containing oestradiol (E_2; 24pg/ml). The pre- or post-treatment histograms represent average of two 10min fractions collected either immediately pre- or post-treatment. $p<0.05$ from respective pre-treatment values (Asterisks; Paired t-test). Numbers in brackets represent number of slices.

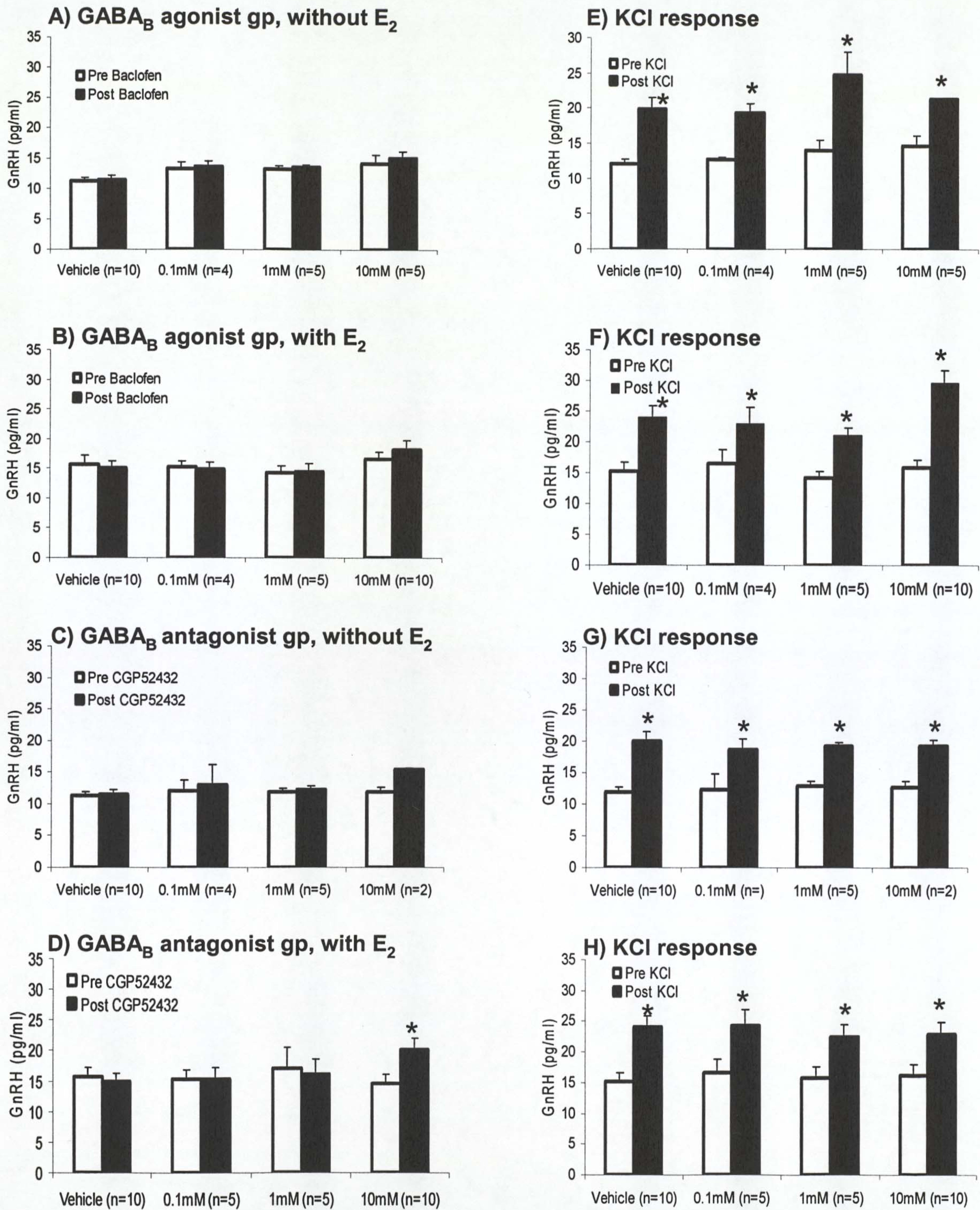


Fig 7.2 Effects of various doses (0.1mM-10mM) of GABA$_B$ agonist (baclofen) or antagonist (CGP52432) and KCl (100mM) challenge upon the release of total immunoreactive GnRH (mean±SEM) from ewe hypothalamic slices perifused with media alone or containing oestradiol (E$_2$; 24pg/ml). The pre- or post-treatment histograms represent average of two 10min fractions collected either immediately pre- or post-treatment. $p<0.05$ from respective pre-treatment values (Asterisks; Paired t-test). Numbers in brackets represent number of slices.

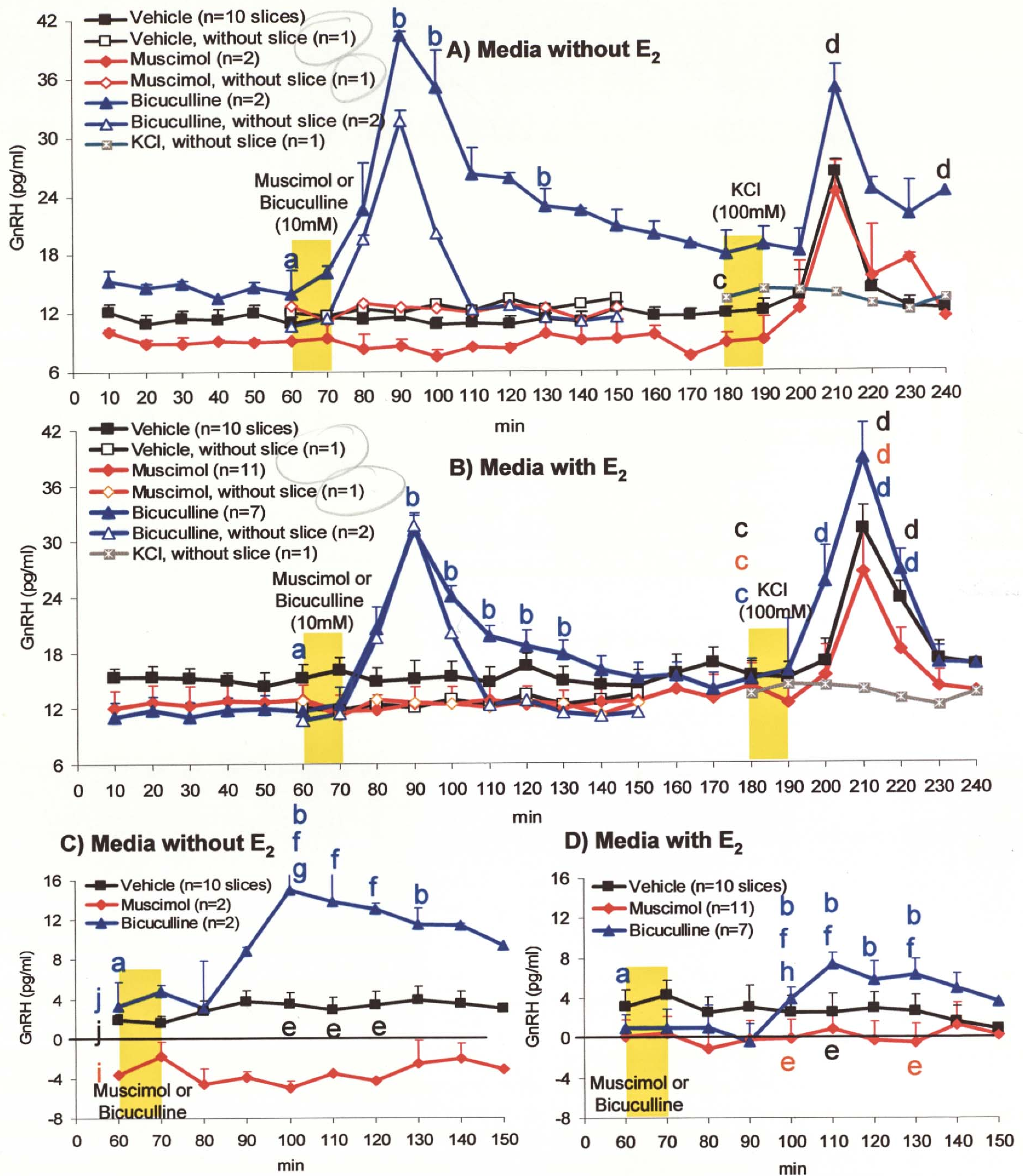


Fig 7.3 Effects of $GABA_A$ agonist (muscimol 10mM) or antagonist (bicuculline 10mM) upon the release of total immunoreactive GnRH from ewe hypothalamic slices perifused with media alone (A,C) or containing oestradiol (B,D; E_2; 24pg/ml). In A and B, compound interaction with GnRH assay suggested by changes in immunoreactive GnRH values in perifusate fractions collected by perifusing muscimol or bicuculline (10mM) through blank chambers. In C and D, net immunoreactive/bioactive GnRH release was obtained by subtracting immunoreactive GnRH values of perifusates collected from blank chambers after exposure to compounds from immunoreactive GnRH values of each hypothalamic slice-containing chamber exposed to the respective compounds. $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f, g *vs* h; Tukey's pairwise comparison). In C, muscimol group values were not compared with other groups due to initial differences in baseline (i *vs* j).

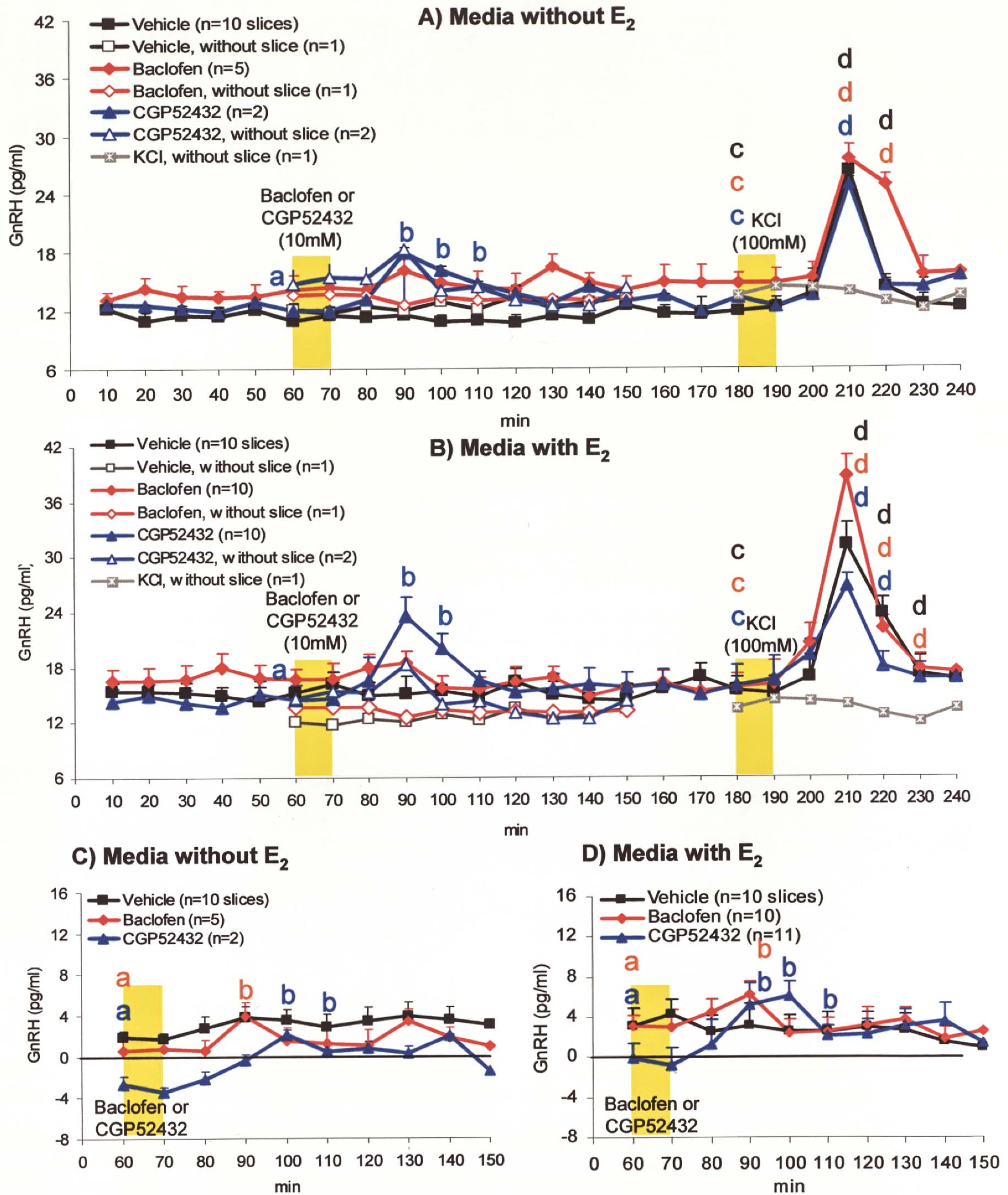


Fig 7.4 Effects of GABA$_B$ agonist (baclofen 10mM) or antagonist (CGP52432 10mM) upon the release of total immunoreactive GnRH from ewe hypothalamic slices perifused with media alone (A,C) or containing oestradiol (B,D; E$_2$; 24pg/ml). In A and B compound interaction with GnRH assay suggested by changes in immunoreactive GnRH values in perifusate fractions collected by perifusing baclofen or CGP52432 (10mM) through blank chambers. In C and D net immunoreactive/bioactive GnRH release was obtained by subtracting immunoreactive GnRH values of perifusates collected from blank chambers after exposure to compounds from immunoreactive GnRH values of each hypothalamic slice-containing chamber exposed to the respective compounds. $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test). No differences were observed between groups.

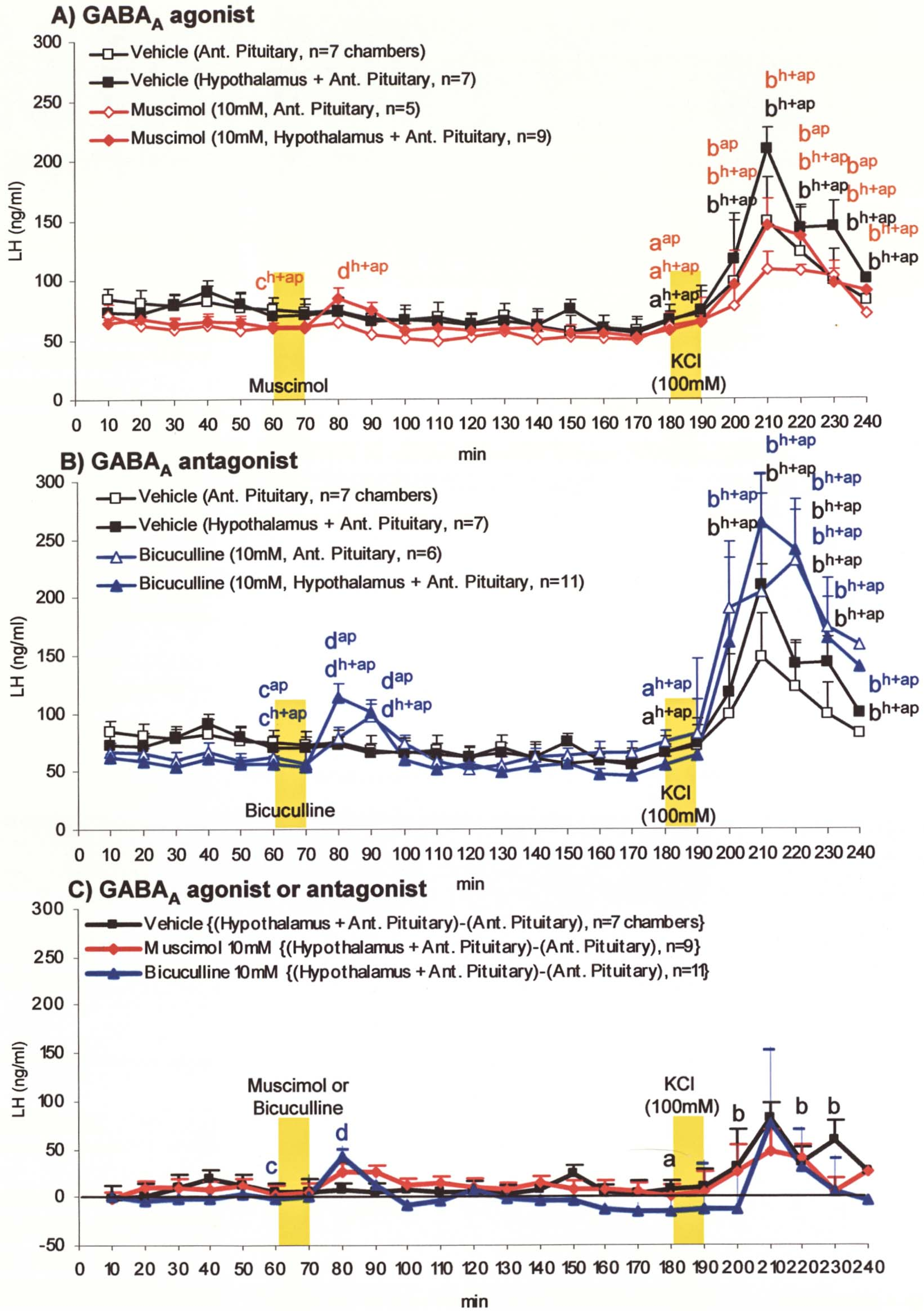


Fig 7.5 Effects of GABA$_A$ agonist (A; muscimol 10mM) or antagonist (B; bicuculline 10mM) or KCl 100mM upon the release of LH from ewe anterior pituitary fragments perifused in absence of E_2, either alone or in series with hypothalamic slices. In C) net LH values showing the effect of bioactive GnRH released in the hypothalamic perifusate on the anterior pituitary fragments. These values were obtained by subtracting, in respective groups, the mean LH responses of pituitary fragments perifused alone from LH values for each chamber where pituitaries were perifused in series with hypothalamic slices. $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test). Letters with superscripts ^{ap} or $^{h+ap}$ indicate chambers with anterior pituitary alone or anterior pituitary in series with a hypothalamic slice, respectively.

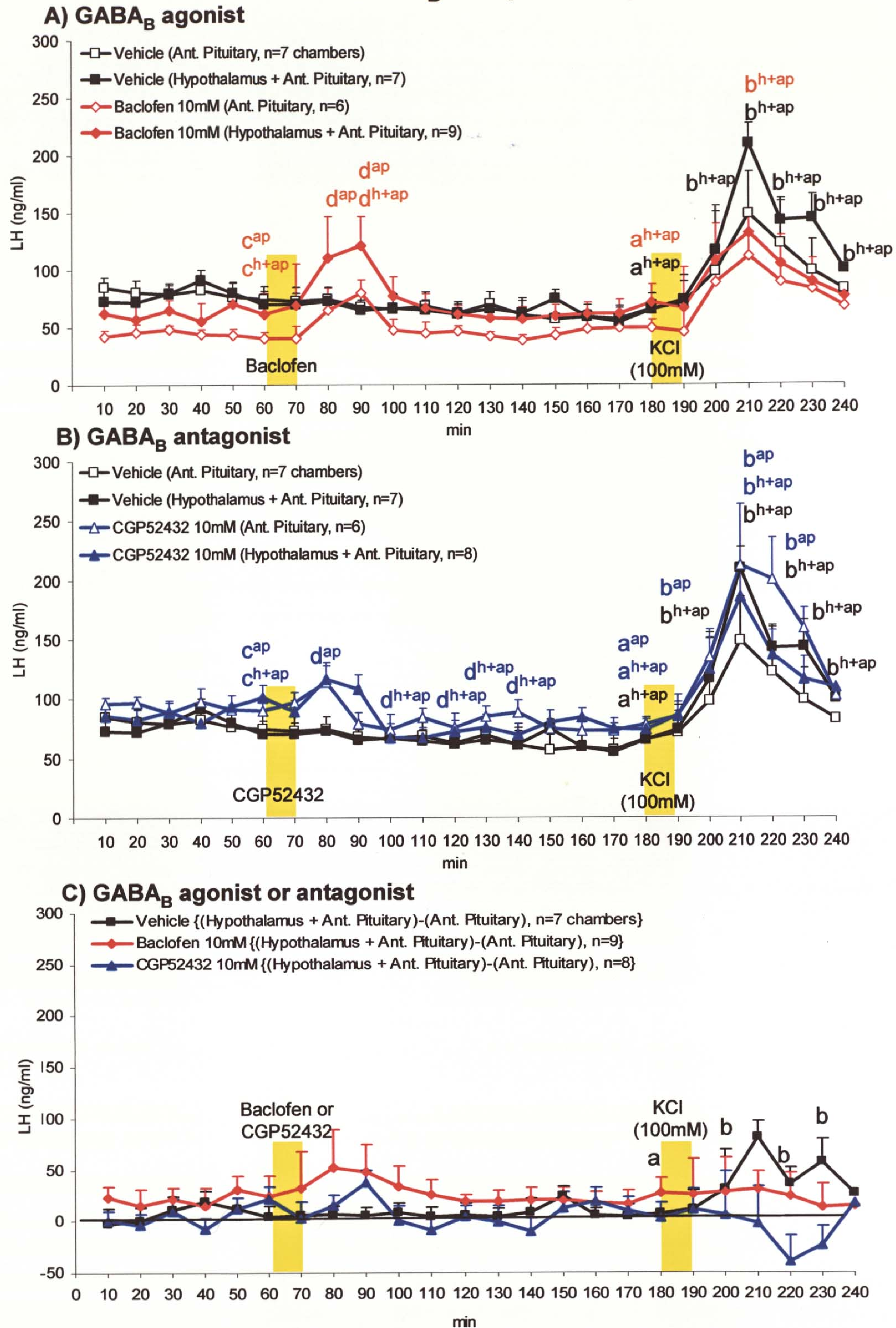


Fig 7.6 Effects of GABA$_B$ agonist (A; baclofen 10mM) or antagonist (B; CGP52432 10mM) or KCl 100mM upon the release of LH from ewe anterior pituitary fragments perifused in absence of E_2, either alone or in series with hypothalamic slices. In C) net LH values showing the effect of bioactive GnRH released in the hypothalamic perifusate on the anterior pituitary fragments. These values were obtained by subtracting, in respective groups, the mean LH responses of pituitary fragments perifused alone from LH values for each chamber where pituitaries were perifused in series with hypothalamic slices. $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test). Letters with superscripts ^{ap} or $^{h+ap}$ indicate chambers with anterior pituitary alone or anterior pituitary in series with a hypothalamic slice respectively.

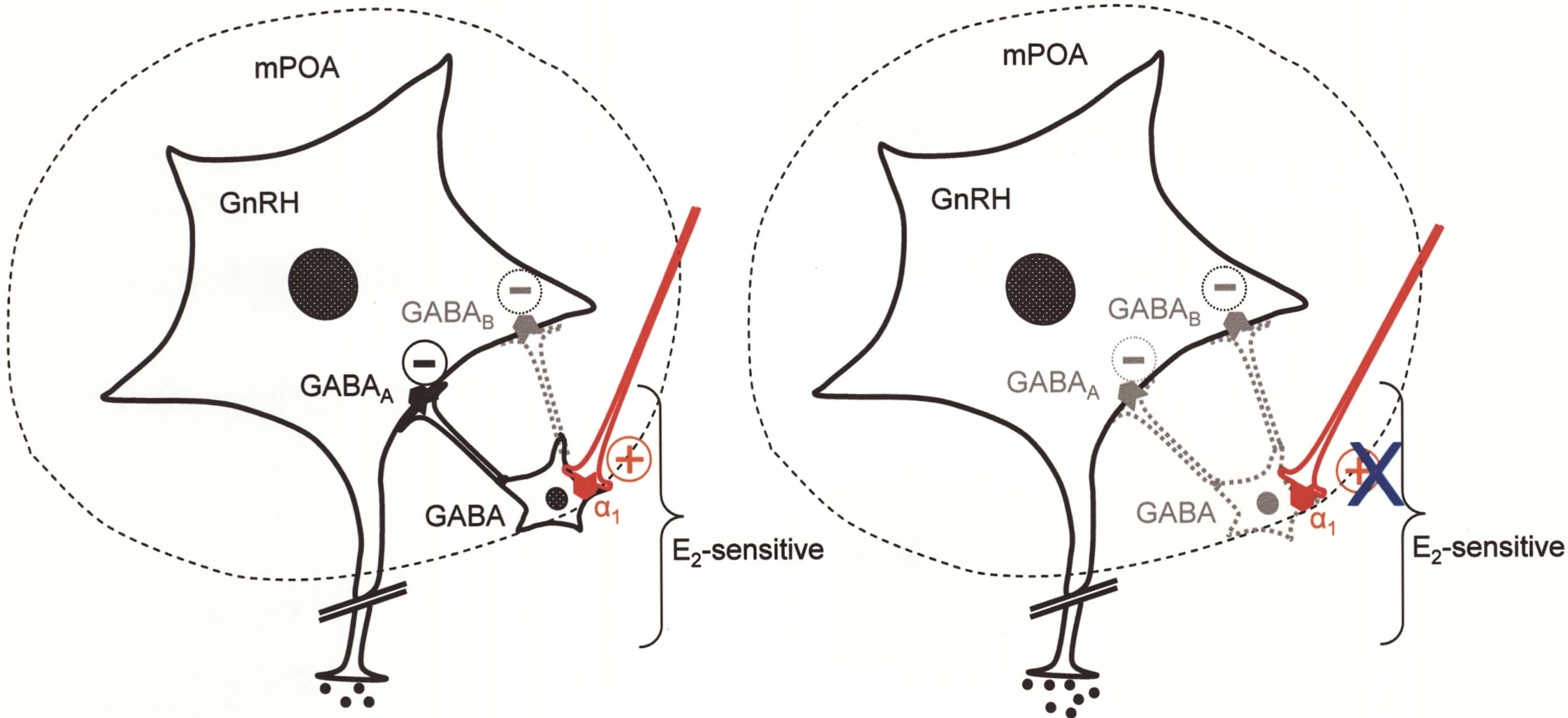


Fig 7.7 Regulation of gonadotrophin releasing hormone (GnRH) neurones by γ-amino butyric acid (GABA) in the absence (A) or the presence of oestradiol (E_2, B). In the medial preoptic area (mPOA), GABA neurones send terminals in close contact with GnRH neurones. In the absence of E_2 (A), noradrenaline via α_1-adrenoreceptors on GABA neurones stimulates GABA to inhibit GnRH release through $GABA_A$ receptors, however, in the presence of E_2 (B), noradrenaline does not simulate GABA release, thus lifting the tonic inhibition of GABA on GnRH neurones. 3V, Third ventricle

Chapter 8

Noradrenergic control of GnRH release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

Noradrenergic control of GnRH release from ewe hypothalamus *in vitro*: sensitivity to oestradiol

Abstract

The present study investigates the influence of α_1-adrenoreceptors on GnRH release *in vitro* and determines whether E_2 modulates α_1-adrenoreceptor and GnRH interaction. Ten minutes after ewe sacrifice, saggital midline hypothalamic slices (from the anterior preoptic area to mediobasal hypothalamus with the median eminence, 2 mm thick, 2 per sheep) were dissected, placed in oxygenated MEM-α at 4°C and within the 2 h were singly perifused at 37°C with oxygenated MEM-α (pH 7.4; flow rate 0.15 ml/min), either with or without E_2 (24 pg/ml). After 4 h equilibration, 10 min fractions were collected for 4 h interposed with a 10 min exposure at 60 min to specific α_1-adrenoreceptor agonist (methoxamine) or antagonist (thymoxamine) at various doses (0.1-10 mM). The α_1-adrenoreceptor compounds interacted with the GnRH assay. Therefore, the effect of α_1-adrenoreceptor compounds on net immunoreactive GnRH release was obtained after subtracting the artefactual increase from appropriate control chambers. The α_1-adrenoreceptor agonist (10 mM) stimulated net immunoreactive GnRH release at 90 min ($p<0.05$; No E_2, n=4: 1.5±0.4 to 3.8±0.5, With E_2, n=4: 4.8±1.1 to 11.9±1.0 pg/ml). However, in slices perifused with media containing E_2 and α_1-adrenoreceptor agonist, net immunoreactive GnRH release remained elevated ($p<0.05$) for at least 60 min.

Furthermore, when anterior pituitary fragments were perifused in series with hypothalamic slices, the stimulatory effect of net bioactive GnRH released by 10 mM α_1-adrenoreceptor agonist resulted in net LH release (n=9, 7.8±12.3 to 36.2±21.6 ng/ml). This confirms that the α_1-adrenoreceptor agonist did stimulate net GnRH release from hypothalamic slices and this was biologically active. At the end of the collection period, all hypothalamic slices or anterior pituitary fragments responded to KCl (100 mM) with GnRH or LH efflux ($p<0.05$). In summary, GnRH release from the hypothalamus is under stimulatory noradrenergic control potentiated by E_2.

Introduction

Associated with the preovulatory GnRH/LH surge during the breeding season, there is an increase in noradrenaline release in the medial preoptic area (mPOA) and the arcuate nucleus-median eminence complex (ARC-ME) in the rat and ewe (Demling *et al.*, 1985; Domanski *et al.*, 1991; Mohankumar *et al.*, 1994; Clarke *et al.*, 1999). In rats, the stimulatory influence of noradrenaline on the GnRH system is mediated via α_1-adrenoreceptors as α-adrenoreceptor antagonists consistently block the oestradiol (E_2)-induced LH surge (Drouva *et al.*, 1982; Coen & Coombs, 1983). In the ME of monkeys, noradrenaline pulses drive GnRH pulses (Terasawa *et al.*, 1988), but no temporal correlation has been observed between noradrenaline in the ovine POA and LH pulses (Robinson *et al.*, 1991; Robinson & Kendrick, 1992a). Also, the inability of an α-adrenoreceptor antagonist to alter GnRH or LH pulse frequency in E_2-treated ovariectomized (OVX) ewes questions any positive correlation in this species (Goodman *et al.*, 1996).

In the rat, α_{1B}/α_{2A}-adrenoreceptors colocalise with GnRH neurones as well as in GT-1 cell-lines (Findell *et al.*, 1993; Lee *et al.*, 1995; Hosny & Jennes, 1998). Electron microscopy reveals synaptic contacts between GnRH neurones and noradrenergic terminals in the rat POA (Leranth *et al.*, 1988a; Chen *et al.*, 1989b), and in the ewe, close contacts have been observed between noradrenergic terminals and a sub population (16-35%) of GnRH neurones in the mPOA (Pompolo *et al.*, 2003a). In an earlier study in the ewe, systemic administration of noradrenaline in the late spontaneous follicular phase stimulates LH secretion, whereas non-specific α-adrenoreceptor antagonists block the LH surge (Narayana & Dobson, 1979a). However, it is necessary to confirm a functional role for the noradrenergic system acting via specific α_1-adrenoreceptors.

Noradrenergic neurones in the ewe brainstem colocalise E_2-receptor-α (ERα) (Simonian *et al.*, 1998; Scott *et al.*, 1999). In rats and ewes, a positive correlation between E_2 concentrations and noradrenergic release in the POA and in the ME suggests that noradrenaline is pivotal in mediating the stimulatory action of E_2 on the GnRH network (Demling *et al.*, 1985; Domanski *et al.*, 1991; Mohankumar *et al.*,

1994). However it is not clear whether E_2 acts within the hypothalamus or only through the brainstem noradrenergic neurones to modulate GnRH release in ewe.

Thus in the present study, we sought to determine any correlation between the noradrenergic and GnRH systems within the hypothalamus under the influence of E_2 using an *in vitro* perifusion system during the breeding season. An *in vitro* approach was essential to avoid any confounding effects of α_1-adrenoreceptor compounds or E_2 on other body systems.

Materials and Methods

Hypothalamic slice and anterior pituitary tissue perifusion in vitro

Perifusions (hypothalamus ± pituitary in series) were performed as described in detail elsewhere (chapter 2). After a 4 h equilibration period, medium effluent fractions were collected for 4 h.

Treatments

Hypothalamic slices alone were perifused with media, with or without E_2 (24 pg/ml, Sigma-Aldrich). After a 60 min collection period, slices were treated for 10 min with vehicle (MEM-α, without E_2, n=10 perifusion chambers; with E_2, n=10), or different doses of a α_1-adrenoreceptor agonist (methoxamine, Sigma-Aldrich; with or without E_2, 0.1 mM, n=2 each; 1 or 10 mM, n=4 each) or antagonist (thymoxamine, Parke-Davis, UK; with or without E_2, 0.1 mM, n=3 each; 1 or 10 mM, n=4 each).

Anterior pituitary fragments alone (vehicle, n=7; methoxamine 10 mM, n=6; thymoxamine 10 mM, n=5) or in series with hypothalamic slices (vehicle, n=7; methoxamine 10 mM, n=9; thymoxamine 10 mM, n=8) were perifused without E_2. At the end of the perifusion, all hypothalamic slices or anterior pituitary fragments were exposed to a depolarizing stimulus of KCl (100 mM) for 10 min after 180 min of perifusate collection.

GnRH assay

GnRH was measured by radioimmunoassay (chapter 2). Sensitivity of the assay was 5.2 pg/ml. Inter- and intra-assay coefficients of variation were 7.7% and 5.8%, respectively. There was no interaction in the GnRH assay with E_2 or KCl, however, there was an interaction with methoxamine. Therefore, empty chambers were run with media perifusate collected during the equilibration period in order to be able to subtract the effects of methoxamine on the assay rather than artefactual GnRH release. This part of experiment was performed in a manner identical to that of the hypothalamic slice perifusions alone.

LH assay

LH was measured by radioimmunoassay (chapter 2). Sensitivity of the assay was 0.3 ng/ml. Inter- and intra-assay coefficients of variation were 7.5% and 8.2%, respectively. There was no interaction in the LH assay with methoxamine, thymoxamine or KCl at the concentrations used in this study.

Statistical analysis

Basal release, with or without E_2, was defined as the mean immunoreactive GnRH release in perifusate fractions collected for 60 min, prior to start of α_1-adrenoreceptor treatment. For dose response studies (Figs 8.1A-D), pre- or post-treatment values were calculated by averaging the total immunoreactive GnRH concentrations (artefactual immunoreactive GnRH, if any, plus net immunoreactive GnRH) of individual chambers at 60 and 70 min for pre-treatment, and at 80 and 90 min for post-treatment. Two time points after treatments were chosen as the mean time of peak response was variable between chambers. Similarly, pre- and post-KCl (Figs 8.1E-H) concentrations of GnRH were calculated by averaging 180 and 190, or 200 and 210 min fractions, respectively. The net immunoreactive GnRH release (Figs 8.2C-D, 60-150 min) was calculated by subtracting the immunoreactive GnRH values of empty chambers (clearly α_1-adrenoreceptor compound interaction in the GnRH assay) from total immunoreactive GnRH values of hypothalamic slice-containing chambers (Figs 8.2A-B). In the perifusion experiments of anterior pituitary fragments

alone, LH was released after exposure to α_1-adrenoreceptor compounds or KCl. Thus, the net LH release (Fig 8.3C), reflecting the effect of net bioactive GnRH released from hypothalamic slices, was calculated by subtracting the mean LH of pituitary-containing chambers from in series pituitary chambers in respective groups (Figs 8.3A-B).

Statistical analysis was performed as described previously (chapter 4).

Results

The mean basal (10-60 min) immunoreactive GnRH released by hypothalamic slices perifused with medium containing E_2 was higher ($p<0.05$, 14.7±0.1 pg/ml, n=18, Fig 8.2B) than slices perifused with medium containing no E_2 (11.2±0.1 pg/ml, n=18, Fig 8.2A).

The effects of various concentrations of methoxamine and thymoxamine (0.1-10 mM) on the release of total immunoreactive GnRH from hypothalamic slices alone are shown in Fig 8.1. Exposure of hypothalamic slices to 10 mM methoxamine stimulated total immunoreactive GnRH release with or without E_2 (Figs 8.1A-B). The pattern of total immunoreactive GnRH release during the post-treatment period (80-150 min) following 10 mM methoxamine or thymoxamine is emphasized in Figs 8.2A and B. Also, the results of artefactual increases in immunoreactive GnRH due to assay interference are shown in Figs 8.2A and B (compound without slice). Exposure to 10 mM methoxamine increased immunoreactive GnRH both from the blank chamber and from the chambers containing hypothalamic slices (Figs 8.2 A-B).

The increase in net immunoreactive GnRH obtained after subtracting the effects of the compounds is represented in Figs 8.2C and D. The elevated concentrations of net immunoreactive GnRH induced by 10 mM methoxamine were maintained up to 150 min, both with (Fig 8.2D) or without E_2 (Fig 8.2C). More net immunoreactive GnRH was released after 10 mM methoxamine than 10 mM thymoxamine in the presence of E_2 (Fig 8.2D).

To confirm that the hypothalamus was secreting net bioactive GnRH, 10 mM methoxamine stimulated LH release when exposed to pituitary alone or with

hypothalamus and pituitary placed in series (Fig 8.3A). However, thymoxamine stimulated LH release only when the hypothalamus and pituitary were placed in series (Fig 8.3B). The net increase in LH calculated by subtracting the LH output of hypothalamus with pituitary placed in series from the pituitary alone is represented in Fig 8.3C, where the stimulatory effect of 10 mM methoxamine on net LH release failed to reach statistical significance (due to large inter-chamber variance).

At the end of perifusions, potassium chloride exposure stimulated immunoreactive/bioactive GnRH or LH confirming that both hypothalamic slices and anterior pituitary tissues were viable (Figs 8.1E-H, Figs 8.2A-B, Fig 8.3).

Discussion

To our knowledge, this is the first evidence for an *in vitro* interaction between ovine noradrenergic and GnRH systems and shows that noradrenergic action is potentiated by E_2. Methoxamine, a selective α_1-adrenoreceptor agonist, enhanced net immunoreactive and bioactive GnRH release from ewe hypothalamic slices *in vitro* confirming a stimulatory influence of noradrenergic systems actually within the hypothalamus on GnRH release. These results substantiate *in vivo* observations that increases in noradrenaline in the POA and in the ARC-ME complex occur in association with the preovulatory GnRH surge (Demling *et al.*, 1985; Domanski *et al.*, 1991; Mohankumar *et al.*, 1994; Clarke *et al.*, 1999). There is also a suggestion in rats and monkeys that noradrenaline stimulates the release of GnRH through an α-adrenoreceptor mechanism in the mediobasal hypothalamus or ME (Negro-Vilar *et al.*, 1979; Nowak & Swerdloff, 1985; Terasawa *et al.*, 1988).

In the present study, it is not clear at which precise sites the predominantly stimulatory influence is exerted. There is a dense network of noradrenergic terminals around the GnRH neurones in the ovine POA (Lehman *et al.*, 1988b; Tillet & Thibault, 1989) but the location of specific adrenoreceptors in the ovine hypothalamus has not yet been examined. In rats, all GnRH neurones in the mPOA have α_1-adrenoreceptors, whereas in the ME, there are very few α_1-adrenoreceptors and they are not associated with GnRH terminals (Hosny & Jennes, 1998). This suggests that

the effects of α_1-adrenoreceptors on GnRH release are not mediated by activation of α_1-adrenoreceptors within the ME but rather within the mPOA.

Importantly, the α_1-adrenoreceptor agonists may mediate E_2 influence on GnRH secretion via actions at sites other than the POA. In ewes, E_2-implants in the ventromedial nucleus (VMN) induce a gonadotrophin surge (Blache *et al.*, 1991; Caraty *et al.*, 1998a), and in the nearby ARC-ME region, there is heavy noradrenergic innervation (Tillet & Thibault, 1989; Rawson *et al.*, 2001). Furthermore, there is a marked increase in noradrenaline release from the ARC-ME at the time of the E_2-induced LH surge (Kaynard *et al.*, 1990; Domanski *et al.*, 1991; Anderson *et al.*, 2001b). Whether E_2 acts through noradrenergic terminals located in the POA and/or the ARC-VMN to augment GnRH release in the ewe can now be specifically tested by *in vitro* injection at precise hypothalamic sites in a minor modification of our perifusion system.

Basal release of GnRH from hypothalamic slices was augmented by the presence of E_2 confirming previous *in vitro* and *in vivo* studies in the ewe (chapter 3) (Moenter *et al.*, 1990). Another important observation from the present study was that α_1-adrenoreceptor agonists further enhance GnRH release in the presence of E_2. Similarly in the rat, a positive correlation exists between E_2 concentrations and noradrenaline release in the POA indicating that noradrenaline has a major role here in mediating the stimulatory action of E_2 on the GnRH system (Demling *et al.*, 1985; Domanski *et al.*, 1991; Mohankumar *et al.*, 1994).

In vivo studies in the ewe also implicate the brainstem in transmitting the actions of E_2 onto the GnRH network since brainstem noradrenergic neurones colocalise ERα, and E_2 rapidly increases *c-fos* expression and electrical excitability of noradrenergic neurones projecting from the brainstem to the POA (Kaba *et al.*, 1983; Clarke, 1995a; Simonian *et al.*, 1998; Scott *et al.*, 1999; Rawson *et al.*, 2001). However, the present study shows that even without further brainstem involvement, noradrenergic and E_2 interaction in the hypothalamus augments the release of GnRH. This could be explained by firstly, E_2-induced enhancement of α_1-adrenoreceptor coupling within the GnRH network (Petitti & Etgen, 1990), and secondly, by E_2-

induced alterations in noradrenergic-GABA interactions in the POA (discussed below).

In the present study, prolongation of net GnRH release by E_2 could occur via an interaction of the noradrenergic system with GABA neurotransmission within the hypothalamus. In rats, there are many GABA neurones in the POA colocalising α_1-adrenoreceptors and receiving noradrenergic terminals from the brainstem (Leranth *et al.*, 1988a; Han *et al.*, 2002). Inhibition of GABA neurones, which suppress GnRH neurones in the ewe POA, provides putative pathway by which noradrenaline influences GnRH neurones (Herbison *et al.*, 1990; Jansen *et al.*, 2003). *In vivo* studies in rats and sheep demonstrate that with high E_2, there is uncoupling of the noradrenaline stimulatory influence upon GABA neurones prior to the LH surge, thus relieving the GnRH neurones from GABA inhibition (Herbison *et al.*, 1990; Robinson *et al.*, 1991; Herbison, 1997a). A similar mechanism could be proposed from the present *in vitro* results. In the presence of E_2, stimulatory influence of α_1-adrenoreceptor agonist increases on GnRH neurones along with the decrease in inhibitory GABA influence through $GABA_A$ receptors (chapter 7, Fig 8.4).

The present study also indicates that α_1-adrenoreceptor agonists can directly increase LH release from ewe anterior pituitary fragments. This observation is in accordance with an earlier rat study in which noradrenaline induced LH release from the pituitary both by increasing GnRH release from the hypothalamic slices and by direct action on the pituitary (Miyake *et al.*, 1983). Furthermore, adrenoreceptors have been detected in rat anterior pituitary gonadotrophs (Swartz & Moberg, 1986; Alarcon *et al.*, 2001). Studying the effect of adrenoreceptor compounds on LH release from pituitary was not the primary objective of this study, although the greater release of LH from in series pituitary fragments confirms that α_1-adrenoreceptor agonist stimulates net bioactive GnRH from the hypothalamus.

Overall, we have demonstrated augmentation of α_1-adrenoreceptor stimulatory influence on GnRH release from ewe hypothalamic slices *in vitro* in presence of E_2, confirming that noradrenaline is an important neurotransmitter system with actions within the hypothalamus that are influenced by E_2. Furthermore, the present study establishes the potential of this perifusion system for the investigation of the GnRH

response to other neurotransmitter agents and their modulators, including steroid hormones.

α_1-adrenoreceptor compounds

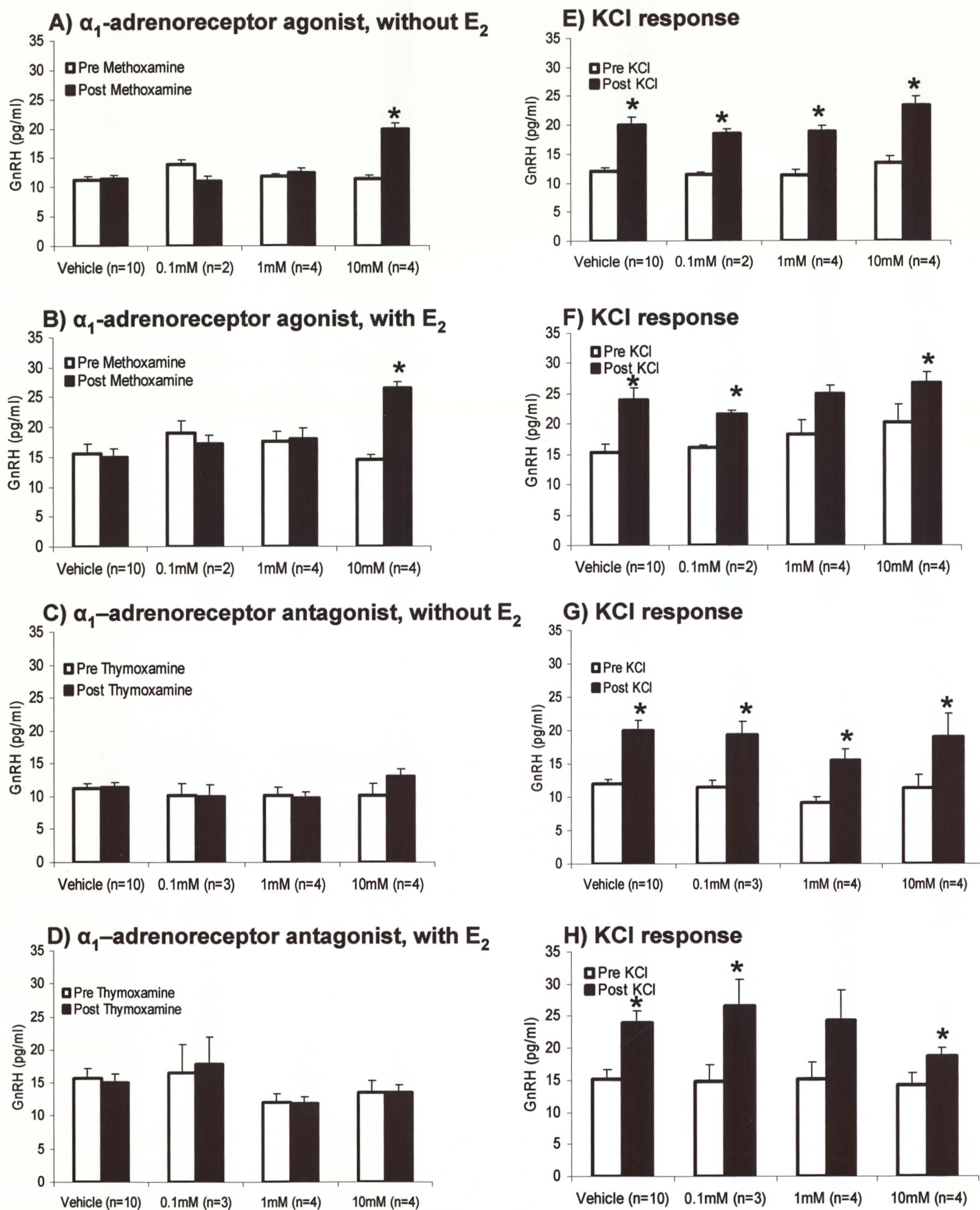


Fig 8.1 Effects of various doses (0.1mM-10mM) of an α_1-adrenoreceptor agonist (methoxamine) or antagonist (thymoxamine) and KCl (100mM) challenge upon the release of total immunoreactive GnRH (mean±SEM) from ewe hypothalamic slices perifused with media alone or containing oestradiol (E_2; 24pg/ml). The pre- or post-treatment histograms represent average of two 10min fractions collected either immediately pre- or post-treatment. $p<0.05$; from respective pre-treatment values (Asterisks; Paired t-test). Numbers in brackets represent number of slices.

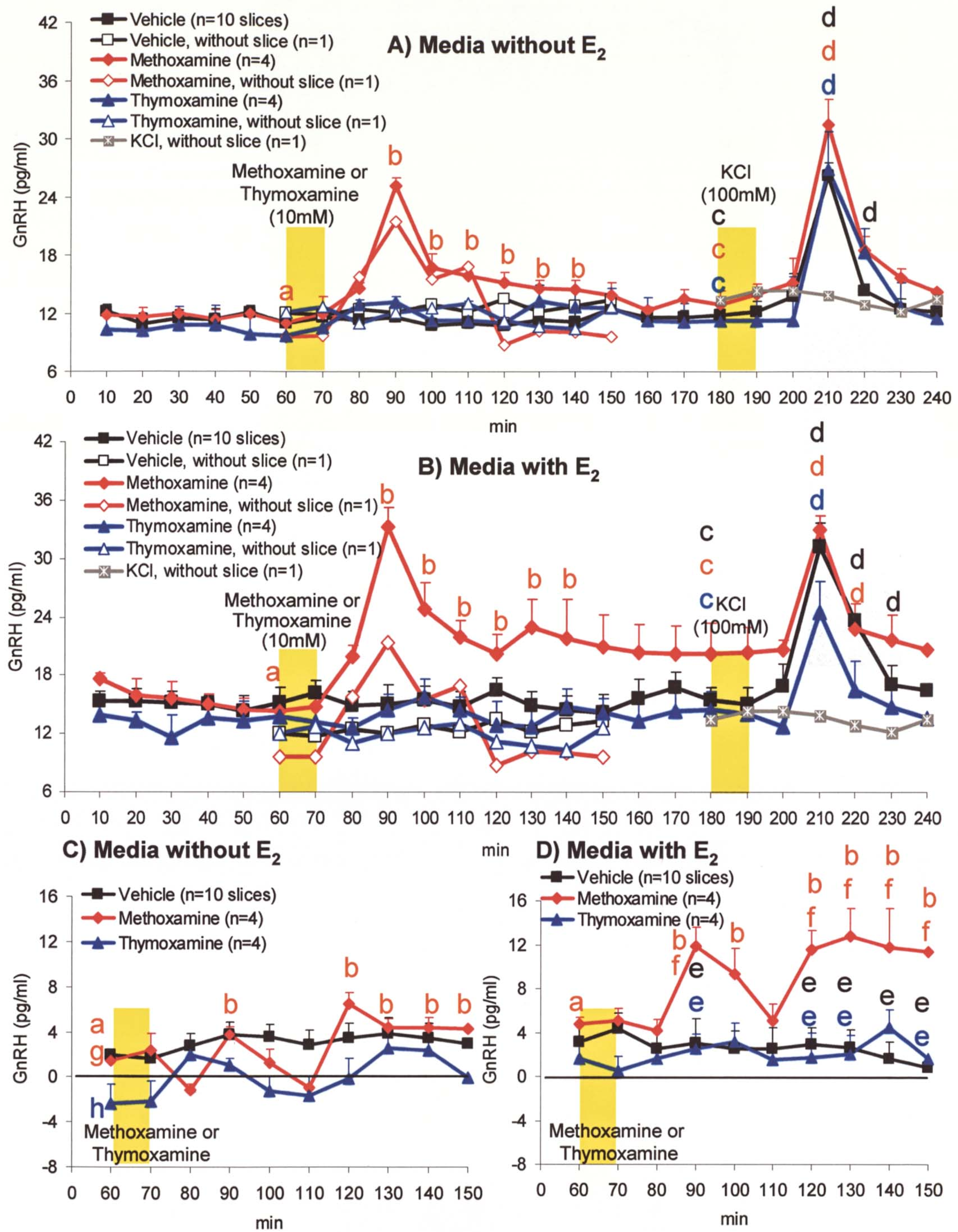


Fig 8.2 Effects of an α_1-adrenoreceptor agonist (methoxamine 10mM) or antagonist (thymoxamine 10mM) upon the release of total immunoreactive GnRH from ewe hypothalamic slices perifused with media alone (A, C) or containing oestradiol (B, D; E_2; 24pg/ml). In A and B compound interaction with GnRH assay suggested by changes in immunoreactive GnRH values in perifusate fractions collected by perifusing methoxamine or thymoxamine (10mM) through blank chambers. In C and D, net immunoreactive/bioactive GnRH release was obtained by subtracting immunoreactive GnRH values of perifusates collected from blank chambers after exposure to compounds from immunoreactive GnRH values of each hypothalamic slice-containing chamber exposed to the respective compounds. $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f, g *vs* h; Tukey's pairwise comparison). In C, thymoxamine group values were not compared with methoxamine group due to initial baseline differences.

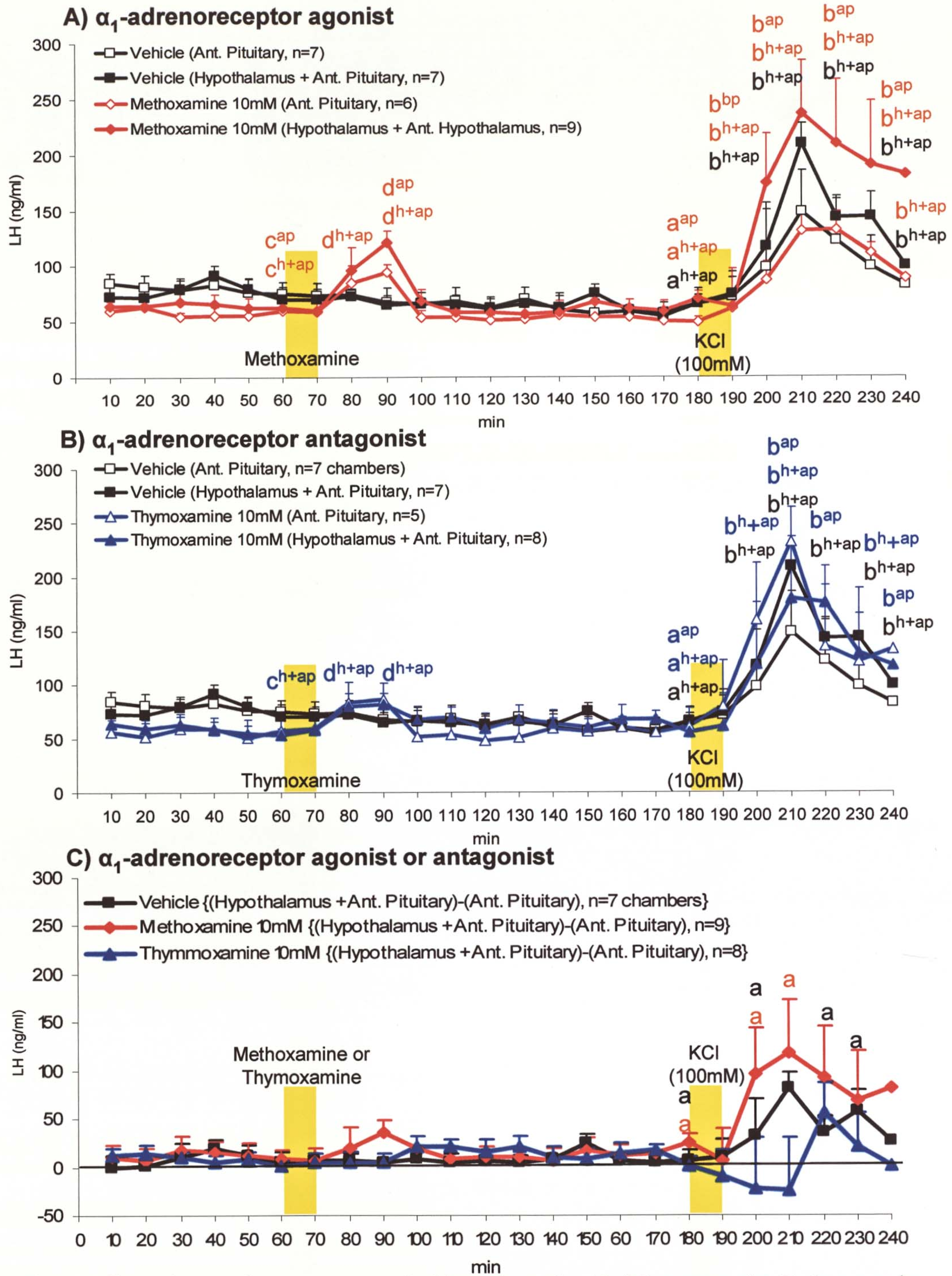
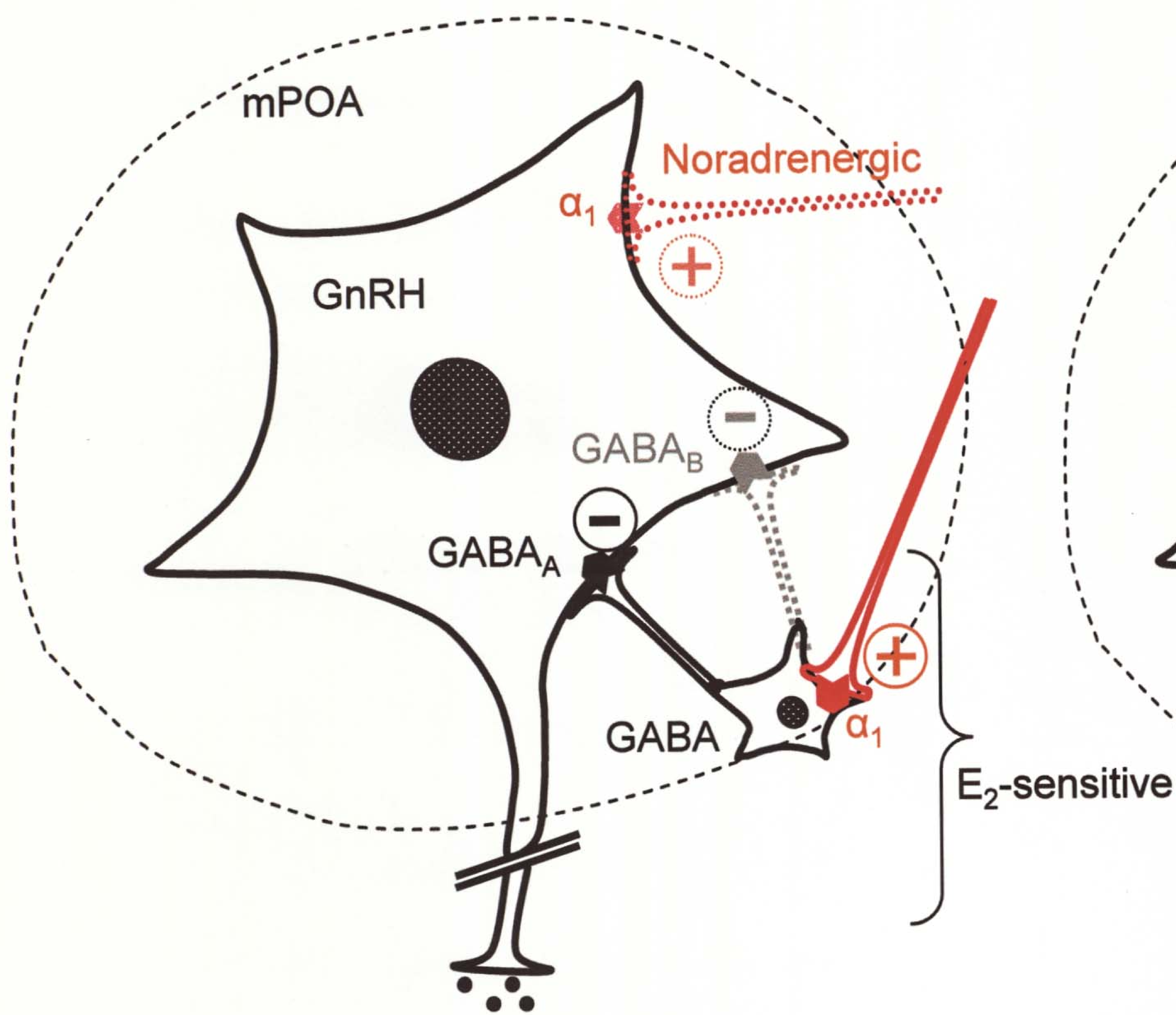


Fig 8.3 Effects of an α_1-adrenoreceptor agonist (A; methoxamine 10mM) or antagonist (B; thymoxamine 10mM) or KCl 100mM upon the release of LH from ewe anterior pituitary fragments perifused in absence of E_2, either alone or in series with hypothalamic slices. In C) net LH values showing the effect of bioactive GnRH released in the hypothalamic perifusate on the anterior pituitary fragments. These values were obtained by subtracting, in respective groups, the mean LH responses of pituitary fragments perifused alone from LH values for each chamber where pituitaries were perifused in series with hypothalamic slices. $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test). Letters with superscripts ^{ap} or $^{h+ap}$ indicate chambers with anterior pituitary alone or anterior pituitary in series with a hypothalamic slice, respectively.

Hypothesis



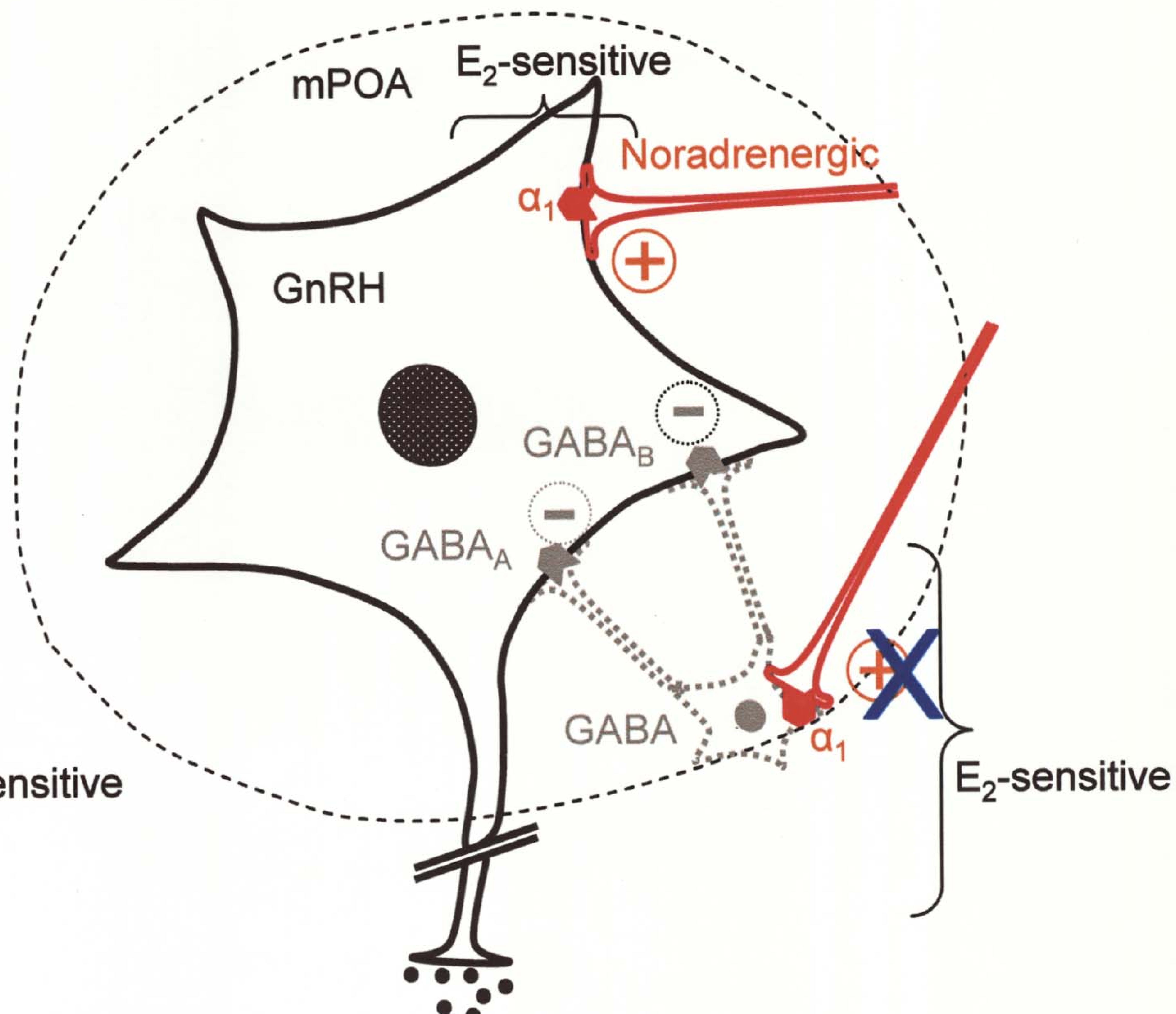


Fig 8.4 Regulation of gonadotrophin releasing hormone (GnRH) neurones by noradrenaline through α_1-adrenoreceptors in the absence (A) or the presence of oestradiol (E_2, B). Noradrenergic terminals are in close contact with GnRH neurones in the medial preoptic area (mPOA). In the absence of E_2 (A), noradrenaline via α_1-adrenoreceptors on GABA neurones stimulates GABA release to inhibit GnRH release through $GABA_A$ receptors, however, in the presence of E_2 (B), noradrenaline does not simulate GABA release, thus lifting the tonic inhibition of GABA on GnRH neurones. Additionally, E_2 facilitates the stimulatory noradrenaline action through α_1-adrenoreceptors on GnRH. 3V, Third ventricle

Chapter 9

Identification of interneuronal connections in the ewe hypothalamus involved in the interaction between stress and reproduction

Identification of interneuronal connections in the ewe hypothalamus involved in the interaction between stress and reproduction

ABSTRACT

This study provides neuroanatomical evidence in the ewe for the suggestion that brainstem noradrenergic and hypothalamic GABA are important modulators of CRH and AVP neurones in the hypothalamic PVN, and β-endorphin neurones in the ARC. Furthermore, possible locations of E_2 involvement in the stress and reproduction interactions are also identified. Thus, double-label immunofluorescence coupled with laser scanning confocal microscopy was used to examine hypothalamic sections from 6 ewes. We found that 62% CRH and 84% AVP cell bodies in the PVN, but none of the β-endorphin cell bodies in the ARC, made close contact with noradrenergic terminals. Furthermore, 81% CRH and 60% β-endorphin but none of the AVP cell bodies were in close apposition to GABA terminals. Although, CRH, AVP or β-endorphin fibres were in the proximity of GnRH cell bodies in the mPOA, no actual close contacts was revealed by confocal analysis; with the exception of a single GnRH neurone in close contact with β-endorphin fibres. Thus, any interaction of CRH, AVP or β-endorphin fibres with GnRH neurone cell bodies in the mPOA would appear to involve other interneurone(s). In the ME, abundant CRH fibres were in the proximity of GnRH fibres providing a route for modulation of release; however, AVP and β-endorphin fibres were not in the same area as GnRH fibres. Brainstem noradrenergic and a subset (32%) of ARC β-endorphin neurones contained ERα, but they were not observed on CRH and AVP neurones in the PVN. The present study provides the basis for a neuroanatomical pathway through which E_2 could modulate the activity of PVN and ARC neurones.

INTRODUCTION

A great deal of evidence indicates that activation of the hypothalamic-pituitary-adrenal (HPA) axis has deleterious effects on hypothalamic-pituitary-gonadal

(HPG) axis activity, and thus reduces fertility of the ewe (Dobson *et al.*, 2003). A similar interaction occurs in rats although in contrast to the ewe activation of the rat HPA is required for facilitation of a normal luteinising hormone (LH) surge in this laboratory species (Kalra, 1993). Thus, it is unwise to wholly extrapolate from the rat and interacting mechanisms must be investigated in other species. Within the hypothalamic paraventricular nucleus (PVN), corticotrophin-releasing hormone (CRH) and parvocellular arginine vasopressin (AVP) neurones play a pivotal role increasing adrenocorticotropin hormone (ACTH) secretion from the pituitary (Smith *et al.*, 2003a). Coincident with an increase in AVP and CRH in portal blood after endotoxin challenge in the ewe, there is suppression of gonadotrophin-releasing hormone (GnRH) release suggesting interactions with the hypothalamus during stress-induced inhibition of GnRH (Battaglia *et al.*, 1998).

Higher up the system, stress provokes a rapid induction of *c-fos* in brainstem noradrenergic neurones (Sawchenko *et al.*, 2000). These neurones send projections to the PVN to form synaptic connections with CRH and AVP neurones (Cummings & Seybold, 1988; Cunningham & Sawchenko, 1988; Michaloudi *et al.*, 1997). Fasting activates noradrenergic projections to the PVN that stimulate CRH release and suppress pulsatile LH secretion in rat (Maeda *et al.*, 1994). All this evidence suggests a connection between activation of brainstem noradrenergic neurones and HPA-HPG interactions in the rat. One aim of the present study is to determine whether similar connections exist to facilitate control of stress and reproduction interactions in the ewe.

Various experiments indicate that γ-amino butyric acid (GABA) has a restraining influence on HPA axis activity (Makara & Stark, 1974; Hillhouse & Milton, 1989). In the rat, half the synaptic terminals on neurones in the PVN contain GABA and have originated from several diffusely distributed sources (Decavel & Van den Pol, 1990; Boudaba *et al.*, 1996). Whether GABA terminals directly interact with CRH and AVP neurones in the PVN in ewe is unknown.

It is also not known whether GnRH cell bodies within the medial preoptic area (mPOA) and/or GnRH fibres in the median eminence (ME) interact with CRH, AVP and β-endorphin neurones to mediate stress-induced suppression of the reproductive

axis. The ME was suggested as a major control site for β-endorphin regulation of the GnRH system in the ewe following infusion of β-endorphin in the ME (Conover *et al.*, 1993). However, others have delayed the onset of the LH surge with an infusion of specific opioid-μ-receptor agonist both in the mediobasal hypothalamus/ME and in the mPOA, suggesting a dual site of control (Walsh & Clarke, 1998). In the rat, CRH inhibits GnRH release only when microinfused into the POA not the PVN, ARC or ME, suggesting the POA as the principal target for CRH action (Rivest *et al.*, 1993). Site-specific CRH/AVP infusion studies have not been carried out in the ewe, but clearly there is a lack of basic knowledge about pathways through which these peptides could suppress GnRH.

The suppression of GnRH by stressors is potentiated in the presence of oestradiol (E_2) in both the rat and ewe (Adam & Findlay, 1998; Li *et al.*, 2003). In the rat, glucoprivation increases oestradiol receptor-α (ERα) expression in the caudal brainstem regions in a dense noradrenergic neurone population (Reyes *et al.*, 2001). In the PVN, CRH neurones contain ERβ in the rat and an E_2-response element exists within the human CRH gene (Vamvakopoulos & Chrousos, 1993; Hahn *et al.*, 2003). Interestingly however, E_2 treatment increases CRH mRNA in the PVN of ovariectomized (OVX) rats but not ewes (Broad *et al.*, 1995; Li *et al.*, 2003). Regarding AVP neurones, E_2 is stimulatory both *in vivo* and *in vitro* in ewes (chapter 3)(Wood *et al.*, 2001). Again, there is a need to investigate if CRH and AVP neurones in the PVN contain ERs, or whether E_2 acts through other ER-positive neurones in the ewe?

Despite the functional importance of noradrenergic and GABA inputs on hypothalamic stress-related neurones in rats, nothing is known about these interactions in the ewe. Neither is it known whether E_2 acts directly on hypothalamic CRH, AVP, β-endorphin neurones or indirectly via noradrenergic neurones from the brainstem. We have recently proposed that several neuronal pathways are involved in the control of normal GnRH release and during stress-induced suppression (Dobson *et al.*, 2003). The present study aims to verify some of these hypotheses, therefore, attempts were made to: 1) characterize the neuroanatomical relationship of noradrenergic and GABA terminals to CRH, AVP and β-endorphin cell bodies, 2) determine whether CRH,

AVP and β-endorphin fibres/terminals interact with the GnRH neuronal system, and 3) identify any colocalisation of ERα in noradrenergic, CRH, AVP or β-endorphin neurones.

MATERIALS AND METHODS

Brain tissue collection procedures, antisera used and immunohistochemistry details are described in Chapter 2. Brainstem and hypothalamus sections were double-labelled with the various antisera combinations as described in Table 9.1.

Sections processed per combination

1) CRH or AVP or β-endorphin with DβH or GAD: one similar section per ewe (n=4) per combination at the locations shown in Fig 9.1B and C. In the PVN, analysis was made from the medial area adjacent to the third ventricle, predominantly parvocellular in the ewe (Hawkins *et al.*, 2001).

2) GnRH with β-endorphin or AVP or CRH: at the level of mPOA or ME, per ewe (n=5) per combination, three sections at 200 μm intervals starting from locations shown in Fig 9.1A and C.

3) ERα with DβH: from the caudal brainstem, starting from obex, at intervals of 1.0 mm, six sections containing the ventrolateral medulla (VLM) and nucleus of solitary tract (NTS) per ewe (n=6).

4) ERα with CRH or AVP: three sections at 280 μm intervals containing PVN around the level shown in Fig 9.1B for each combination per brain (n=6).

5) ERα with β-endorphin: two sections (at 400 μm intervals) containing the ARC from each ewe (n=4; Fig 9.1C).

Light microscopy

Used for identification of double-fluorescent neurones (ERα with DβH or CRH or AVP or β-endorphin) and to assess the number of GnRH neurones near to, or in the area of, dense CRH, AVP or β-endorphin fibres.

Laser Scanning Confocal Microscopy (LSCM)

Possible interneuronal close contacts were examined in the parvocellular PVN, ARC, mPOA and in the ME. Details of LSCM microscopy are described in chapter 2.

Analysis

Confocal data are presented as percentages of the cell bodies in close contact with terminals out of the total number of cell bodies examined in various combinations. ERα data are expressed as mean percentage (±SEM) of total double-labelled neurones in all the sections of each ewe processed for each combination.

RESULTS

Representative confocal images are presented as projections of stacks of optical slices to show the density of innervation by immunoreactive terminals to the areas with neuronal cell bodies of interest. Close contacts are shown in single 0.46 μm optical plane images inserts (Fig 9.2-9.4).

Are noradrenergic or GABA terminals in close contact with CRH or AVP cell bodies in the PVN?

Noradrenergic terminals were in close contact with 62% CRH and 84% AVP cell bodies (Fig 9.2A and B, Table 9.2). Dense GABA innervation of this area made contact with 81% CRH cell bodies (Fig 9.2C, Table 9.2). However, although many GABA terminals were near AVP cell bodies, none made close contact (Fig 9.2D inserts 1-3; Table 9.2).

Are noradrenergic or GABA terminals in close contact with β-endorphin cell bodies in the ARC?

Despite the presence of many noradrenergic terminals in the ARC, none made close contact with β-endorphin cell bodies (Fig 9.3A insert); however, around 60% β-endorphin cell bodies were in close contact with GABA terminals (Fig 9.3B insert; Table 9.2).

Are β-endorphin, CRH or AVP terminals in close contact with GnRH cell bodies in the mPOA and supraoptic nucleus (SON)?

Only 10 of 110 GnRH cell bodies examined in the mPOA appeared to make contact with β-endorphin terminals under light microscopy. Confocal analysis of these, and another 10 not showing contact under light microscopy, revealed close contact of only one GnRH cell body with a β-endorphin terminal (Fig 9.4). In addition, out of 150 GnRH cell bodies examined in the mPOA, only five GnRH cell bodies were near to CRH terminals under light microscopy, but when investigated at the confocal level none were in close contact (image not shown). Furthermore, no GnRH cell bodies were observed to receive any AVP terminals in the mPOA.

In the sections examined, around 15 GnRH neurones were located in the SON associated with a dense AVP neurone population but there were no close contacts with AVP terminals (image not shown).

Are CRH, AVP or β-endorphin fibres close to GnRH fibres in the ME?

CRH fibres were close to GnRH fibres in the external zone of ME (Fig 9.5A). However, the AVP fibres in the ME had no association with GnRH fibres (Fig 9.5B). Scattered β-endorphin neurones and fibres were located in the internal zone of the ME, but there was no evidence of association between β-endorphin and GnRH fibres (Fig 9.5C).

Do ERα colocalise with noradrenergic neurones in the caudal brainstem, CRH or AVP neurones in the PVN or β-endorphin neurones in the ARC?

In VLM and NTS sections of the caudal brainstem, noradrenergic neurones contained ERα (Fig 9.6); however precise quantification was difficult due to technical problems. In the PVN, CRH or AVP neurones did not contain ERα (image not shown). Analysis of the ARC revealed that out of a total of approximately 180 β-endorphin neurones examined per ewe, 32±3% contained ERα (Fig 9.7).

DISCUSSION

The present study provides evidence for a close neuroanatomical relationship between noradrenergic terminals and CRH or AVP cell bodies in the PVN, and between GABA terminals and CRH or β-endorphin cell bodies in the PVN and ARC, respectively. There is an association between CRH and GnRH fibres but only in the ME. Neither CRH nor AVP neurones contain ERα, however, to modulate the stress axis, E_2 may act through ERα containing noradrenergic neurones in the brainstem and/or β-endorphin neurones in the ARC. It is noted that LSCM is not sufficiently detailed to identify true synapses between neurone cell bodies, but does provide anatomical support for a possible functional relationship between the contacting terminal and the neurone cell body.

Noradrenergic terminals closely contact CRH and AVP cell bodies in the PVN

The close contact of noradrenergic terminals with many CRH and AVP cell bodies in the ewe concurs with data from other species. Noradrenergic innervation of the parvocellular division of the PVN, originating from the VLM, NTS and locus coeruleus (LC) regions of brainstem, synapses with CRH and AVP neurones (Cummings & Seybold, 1988; Cunningham & Sawchenko, 1988; Ginsberg *et al.*, 1994; Michaloudi *et al.*, 1997). A similar pathway may exist for the ewe as no noradrenergic cell bodies were observed throughout the hypothalamus in the present study. This modifies the conceptual model described earlier for the ewe where noradrenergic input from the mPOA to the PVN was hypothesised (Dobson *et al.*, 2003). There is substantial evidence of a functional role for noradrenergic input to PVN neurones in the rat but little is known for the ewe. Increased noradrenaline in the PVN stimulates CRH and AVP transcription in the rat (Itoi *et al.*, 1999; Haisenleder, 2000; Cole & Sawchenko, 2002) and intracerebroventricular noradrenaline increases CRH and AVP release (Plotsky *et al.*, 1989; Liu *et al.*, 1991).

During stress, these brainstem noradrenergic terminals have a major role conveying messages to PVN neurones (Ericsson *et al.*, 1994; Li *et al.*, 1996). As an example, destruction of noradrenergic cell bodies in the VLM, NTS and LC by

retrograde neurotoxin administered to the PVN impairs CRH and *c-fos* mRNA expression after glucoprivation and reverses prolongation of oestrous cycle length. This indicates that noradrenergic cell groups are involved in both stress-induced HPA activation and alteration of reproductive function in rats (I'Anson *et al.*, 2003; Ritter *et al.*, 2003).

GABA terminals closely contact CRH, but not AVP, cell bodies in the PVN

The close contact between GABA terminals and the majority of CRH cell bodies provides a route by which GABA can inhibit the HPA in the ewe. Ultrastructural studies in the rat revealed that GABA connections represent one third of all synaptic input that terminates on CRH neurones (Miklos & Kovacs, 2002).

As the antibody used in the present study only detected GABA terminals, it was not possible to confirm whether the GABA cell bodies were within the PVN or elsewhere. However, in addition to the rostral part of the PVN itself, the dorsomedial and anterior hypothalamus, POA and bed nucleus of the stria terminalis (BNST) are all potential sources of direct GABA projections to the PVN (Boudaba *et al.*, 1996). Functionally, the inhibitory GABA control of the HPA axis occurs principally via $GABA_A$ receptors (chapter 4) (DiMicco *et al.*, 1996; Helmreich *et al.*, 1998; Kovacs *et al.*, 1998).

In the present study, AVP cell bodies in the parvocellular subdivision of the PVN were not in close contact with GABA terminals, though many were nearby. However, there is evidence for GABA innervation of AVP neurones in the rat PVN (Decavel *et al.*, 1989). Furthermore, in the rat PVN, there are $GABA_{A \text{ and } B}$ receptors on AVP neurones and our *in vitro* studies suggest an inhibitory GABA control of AVP release (chapter 4)(Fenelon & Herbison, 1995; Fenelon *et al.*, 1995; Slugg *et al.*, 2003; Li & Stern, 2004). Also, in the rat parvocellular AVP expression is more sensitive to inhibition by GABA than CRH expression (Bali & Kovacs, 2003). In the absence of neuroanatomical evidence for a direct interaction between GABA terminals and AVP cell bodies, GABA might inhibit AVP neurones by diffusion. Alternatively, if only parvocellular AVP neurones are the target for GABA, it is possible that we over-looked parvocellular AVP cell bodies with close GABA contacts as these neurones

constituted only 4-5% of the 1200 AVP cell bodies examined in the ewe PVN (Chapter 10). Furthermore, we examined only AVP cell bodies and not AVP axons/dendrites, where close contact with GABA terminals may occur.

GABA, but not noradrenergic, terminals are in close contact with β-endorphin cell bodies in the ARC

The terminals of GABA neurones are in close contact with β-endorphin cell bodies in the ARC, suggesting that GABA could restrain β-endorphin neurones. Indeed, in the rat GABA terminals synapse with β-endorphin neurones in the ARC which in turn project to the PVN (Baker & Herkenham, 1995). The inhibitory effects of opioids on CRH release are well known and removal of opiate influence evokes CRH mRNA expression, as well as CRH and AVP release (Plotsky, 1986; Lightman & Young, 1988; Alexander & Irvine, 1995; Hockings *et al.*, 1995; Janssens *et al.*, 1995). Thus, stimulation by GABA terminals in the ARC may lead to activation of CRH neurones in the PVN through disinhibition, i.e. by inhibiting β-endorphin neurones. Indeed, after insulin-induced hypoglycaemia, β-endorphin activity was suppressed in the ovine ARC concurrently with increased CRH neurone activity in the PVN (Chapter 10).

There were no close contacts between noradrenergic terminals and β-endorphin cell bodies in the ARC, despite the presence of abundant noradrenergic terminals around the β-endorphin cell bodies. The extensive noradrenergic innervation, of unknown origin, has been previously reported in the ovine ARC (Tillet & Thibault, 1989). The absence of close contact suggests that direct interplay between noradrenergic and β-endorphin neurones does not occur in this region.

Overall, β-endorphin neurones may not be stimulated by noradrenergic neurones but could be restrained by GABA neurones.

CRH, AVP and β-endorphin terminals have little or no close contact with GnRH cell bodies in the mPOA

By observing the absence of close contact between CRH terminals and GnRH cell bodies in the mPOA, we confirm recent observations in the rat (Hahn *et al.*, 2003),

but refute earlier results (MacLusky *et al.*, 1988). However, in humans, there are abundant juxtapositions between CRH terminals and GnRH neurones in the ARC, a region that contains very few GnRH cell bodies in the ewe (Lehman *et al.*, 1988a; Dudas & Merchenthaler, 2002). We observed occasional CRH cell bodies in the mPOA (2-6 per section), but the intensity of the staining was not always consistent between animals; therefore no conclusions could be drawn.

Rare AVP fibres were also present in the mPOA close to GnRH neurones but there was no interaction with GnRH cell bodies. Even GnRH cell bodies in the anterior SON, which has a dense AVP neurone population, failed to interact with AVP terminals. This is in contrast to the only other report in which AVP terminals have been reported to synapse on GnRH neurones in the SON (Thind *et al.*, 1991).

In the present study, there were abundant β-endorphin terminals around GnRH cell bodies in the mPOA. However, we observed only one close contact of β-endorphin input to a GnRH cell body in contrast to another recent confocal study (Jansen *et al.*, 2003). The reason for this discrepancy is not clear as similar confocal methods were used although GnRH and β-endorphin antisera were different, however subtle factors such as breeding status may be important.

Overall, it appears that there may be distinct neuroanatomical differences between species concerning CRH, AVP and β-endorphin interactions with GnRH cell bodies. Clearly more studies are required.

In the absence of close contacts observed in the ewe in the present study, interneurone(s) may be involved in stress-induced inhibition of GnRH neurones particularly through the abundant CRH or β-endorphin terminals in the mPOA. Intriguingly, in the rat administration of CRH suppressed GnRH, but only when administered to the mPOA, the area where GnRH neurones have neither close contact with CRH terminals nor CRH type-1 receptors (Rivest *et al.*, 1993; Hahn *et al.*, 2003). In addition, an opioid receptor agonist infusion in the mPOA delayed the LH surge (Walsh & Clarke, 1998). The identity of any interneurones involved needs to be investigated. One probable candidate is the GABA neurone population, that is present in the mPOA and sends terminals to GnRH cell bodies in this area (Pompolo *et al.*, 2003a). Whether CRH, AVP or β-endorphin terminals innervate GABA neurones that

in turn may mediate any messages to GnRH neurones needs to be determined. The present study used GnRH and GAD antisera from the same species so that it was not possible to investigate close GABA contacts with GnRH cell bodies/terminals. Secondly, the GAD antibody only identified GABA axons/terminals, not GABA cell bodies, therefore, we were unable to identify close contacts between CRH, AVP or β-endorphin terminals with GABA cell bodies in the mPOA.

CRH, but not AVP or β-endorphin, fibres are close to GnRH fibres in the ME

There was a clear association between CRH and GnRH fibres in the external zone of ME in the present study. In the ewe, the majority of CRH fibres in the external zone of the ME originate from the PVN, suggesting that activation of CRH cell bodies in the PVN may be involved in the stress-induced suppression of GnRH release at the level of ME in this species (Swanson *et al.*, 1983). The present results concur with *in vitro* studies in which CRH inhibited GnRH release from the isolated rat median eminence; this effect was also reversed by a CRH antagonist, all indicating that the mPOA is not essential for CRH modulation of GnRH output (Gambacciani *et al.*, 1986). In addition, direct application of CRH to the ME *in vivo* suppresses LH release in some but not all studies (Rivest *et al.*, 1993; Ortega *et al.*, 1994; Frias *et al.*, 1997).

There was no evidence for AVP or β-endorphin fibres coming close to GnRH fibres as the majority of the AVP or β-endorphin fibres were in the internal zone and GnRH fibres were in the external zone of the ME. To our knowledge, this is the first study in any species regarding anatomical interaction between AVP and GnRH fibres in the ME. However, we were unable to support the only existing report of synaptic interactions between β-endorphin and GnRH terminals in the ME of monkeys (Thind & Goldsmith, 1988). Thus, it is unlikely that β-endorphin interacts with GnRH terminals in the ME to suppress GnRH release in the ewe. In spite of this, GnRH release is suppressed by β-endorphin administration through push-pull cannula into the ME in this species (Conover *et al.*, 1993). The reason for this discrepancy remains unclear although ORμ have been reported in the external zone of the rat ME (Mansour *et al.*, 1995).

ERα colocalises with noradrenergic neurones in the brainstem and β-endorphin neurones in the ARC, but not with CRH and AVP neurones in the PVN

Noradrenergic neurones were positive for ERα in the caudal brainstem; however we were unable to accurately quantify the precise number of neurones due to technical problems. Previous studies in the ewe observed similar ERα colocalisation although one study counted 14-25% noradrenergic neurones containing ERα in the caudal most VLM and NTS regions, while others observed 80-90% (Simonian *et al.*, 1998; Scott *et al.*, 1999). Neurones from the same regions project to the POA and may mediate actions of E_2 on GnRH release (Rawson *et al.*, 2001). However, nothing is known in the ewe about the functional role of these ERα positive noradrenergic neurones in mediating stress responses in the PVN.

In the present study, we found more (32%) β-endorphin neurones containing ERα in the ARC compared to 15-20% in a previous study (Lehman & Karsch, 1993). This subset of β-endorphin neurones may be important for relaying E_2 signals to GnRH neurones. During the follicular phase of the ovine oestrous cycle, β-endorphin concentrations increase in the ME and this prevents the premature activation of GnRH neurones at times other than immediately before the onset of GnRH surge (Domanski *et al.*, 1991; Conover *et al.*, 1993; Dobson *et al.*, 2003). However, the role of these β-endorphin neurones that colocalise ERα during stress-induced suppression of GnRH release is not known.

We did not observe CRH or AVP neurones with ERα in the PVN, suggesting that these populations are not directly influenced by E_2. The recently identified ERβ, but not ERα, colocalises with both CRH and AVP neurones in the PVN of rats and may ERβ suppress HPA responses (Laflamme *et al.*, 1998; Hahn *et al.*, 2003; Isgor *et al.*, 2003a; Isgor *et al.*, 2003b). Unfortunately, antisera for detection of ERβ in the sheep brain are not currently available.

CONCLUSIONS

The present study demonstrates that CRH and AVP cell bodies in the parvocellular portion of the PVN are in close contact with noradrenergic terminals, but

that β-endorphin cell bodies in the ARC do not receive noradrenergic terminals. Furthermore, close contact occurs between GABA terminals and CRH or β-endorphin, but not AVP, cell bodies. Downstream, there is a close relationship between CRH and GnRH fibres in the ME, but, we found no evidence for a direct effect of CRH, AVP and β-endorphin terminals on GnRH cell bodies in the mPOA. There were no ERα in CRH and AVP cell bodies in the PVN suggesting that E_2 is not directly involved in regulation of CRH and AVP neurones, though E_2 possibly interacts with noradrenergic neurones in the brainstem and β-endorphin neurones in the ARC to modulate HPA axis activity.

Based on our detailed description of interneuronal connections in the ewe hypothalamus, we propose a refined hypothesis for the neuroanatomical framework by which stress and reproduction interact in the ewe (Fig 9.8). From the brainstem, noradrenergic neurones containing ERα could mediate E_2-potentiated stress effects through stimulation of CRH and AVP neurones in the PVN. Also within the PVN, GABA interaction with CRH neurones could restrain activation of the HPA axis. In the ARC, GABA may restrain β-endorphin neurones, thereby removing the opioid inhibition on CRH and/or AVP neurones in the PVN, leading to net excitatory drive. Finally, from the PVN, CRH terminals projecting to the ME could inhibit GnRH secretion by inhibiting the release mechanisms of GnRH secretion from terminals. All this evidence suggests the substantial role of PVN neurones in HPA activation and ultimately GnRH suppression, however, effort is required to determine the involvement of other feedback mechanisms through various hypothalamic and brainstem nuclei (Fig 9.8; chapters 10 and 11).

Table 9.1 Secondary antisera used in various combinations of primary antisera

Area	Primary antisera combination	Primary antisera (a fluorescent tag conjugated to secondary antisera)	
PVN	CRH or AVP with DβH or GAD	CRH or AVP (Anti-rabbit Cy_3)	DβH or GAD (Anti-mouse FITC)
ARC	β-endorphin with DβH or GAD	β-endorphin (Anti-rabbit FITC)	DβH or GAD (Anti-mouse Cy_3)
mPOA or ME	GnRH with CRH or AVP or β-endorphin	GnRH (Anti-mouse FITC)	CRH or AVP or β-endorphin (Anti-rabbit Cy_3)
Brainstem or PVN or ARC	ERα with DβH or CRH or AVP or β-endorphin	ERα (Anti-mouse Cy_3)	others (Anti-rabbit FITC)

Table 9.2 The percentage of CRH and AVP cell bodies in the PVN and β-endorphin cell bodies in the ARC receiving close contacts from noradrenergic (DβH) or GABA (GAD) terminals as determined by laser scanning confocal microscopy

Area	Neurone cell body - immunoreactive input	Number of cell bodies receiving close contacts out of total examined	% cell bodies with close contacts
PVN	CRH - DβH	23/34	62
PVN	AVP - DβH	38/45	84
PVN	CRH - GAD	34/42	81
PVN	AVP - GAD	0/55	0
ARC	β-endorphin - DβH	0/45	0
ARC	β-endorphin - GAD	29/48	60

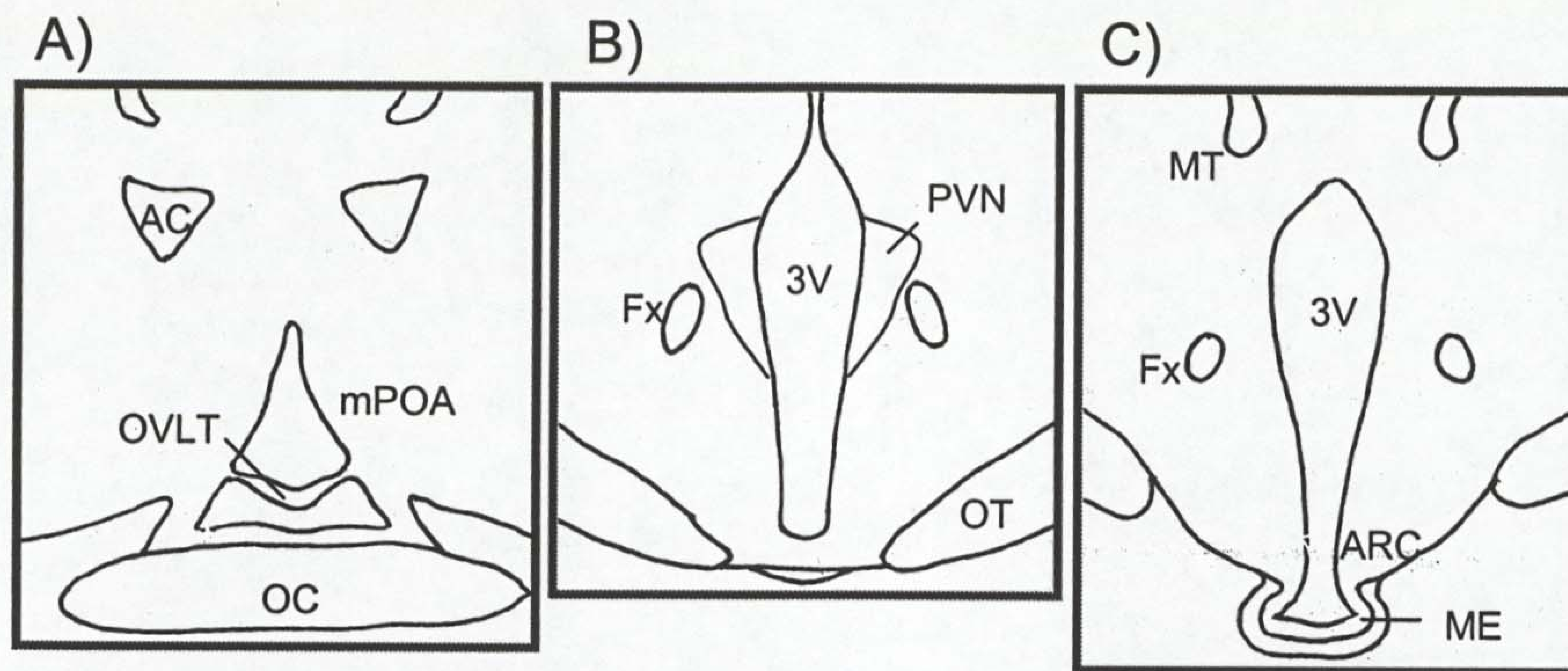


Fig 9.1 Drawings of coronal sections of the ewe hypothalamus showing planes examined for each hypothalamic nucleus: A) the medial preoptic area (mPOA); B) parvocellular subdivision of paraventricular nucleus (PVN); C) arcuate nucleus (ARC) and the median eminence (ME). Sections from these locations were utilized to investigate interneuronal close contacts. AC, anterior commissure; Fx, fornix; MT, mamillothalamic tract; OC, optic chiasm; OT, optic tract; OVLT, organum vasculosum of lamina terminalis; 3V, third ventricle

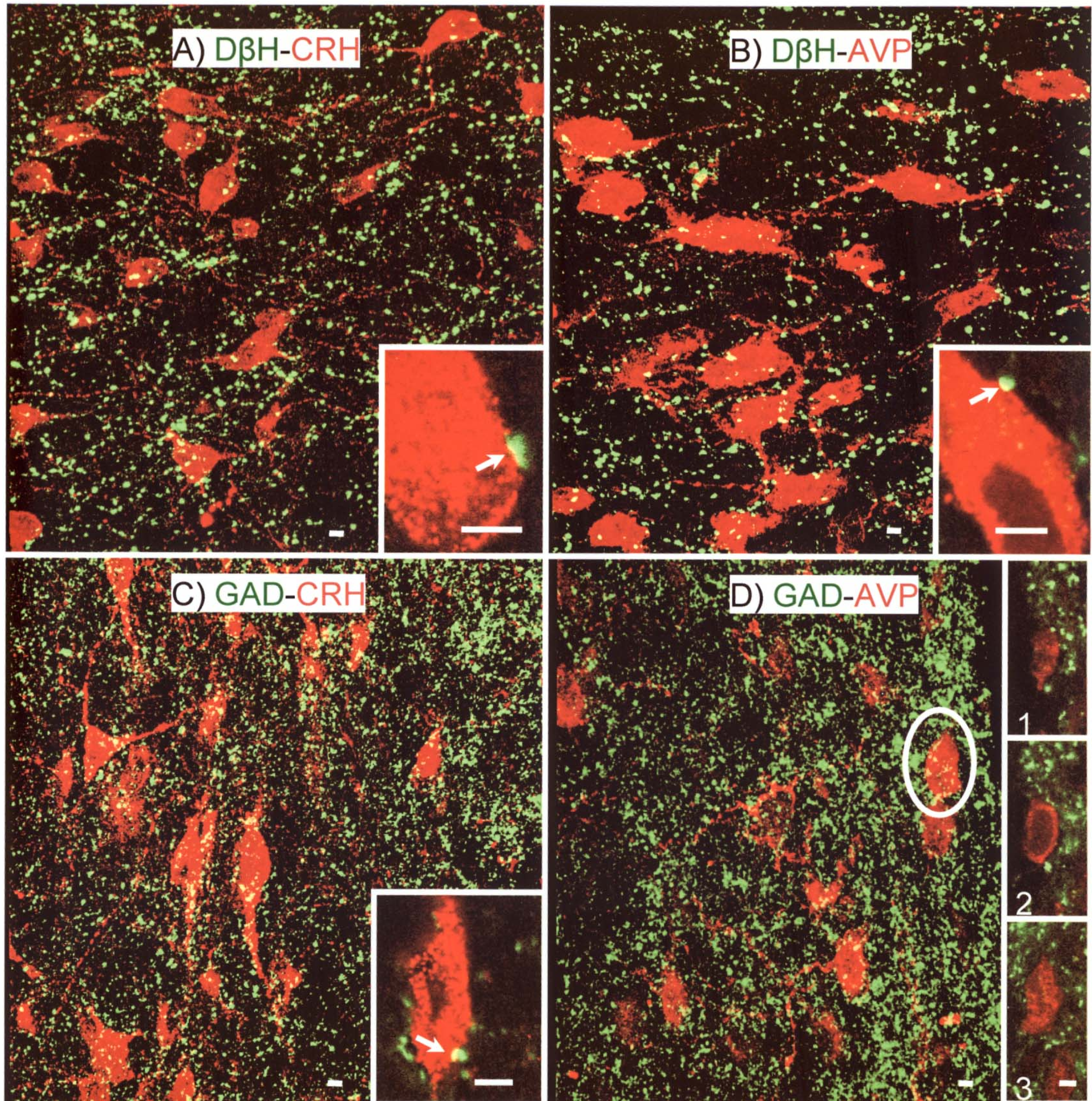


Fig 9.2 Confocal images of noradrenergic (DβH) and GABA (GAD) terminals (green), and CRH and AVP cell bodies (red) in the parvocellular PVN. The stacked confocal images (A, B, C and D) show dense DβH and GAD innervation of the PVN, while close contacts are shown in the individual images in a series of 0.46μm optical sections (Inserts). Arrows in the inserts indicate apparent close contacts. Colocalisation of pixels results in yellow in the inserts, however, yellow in the larger (stacked) images is not always indicative of close contacts; merely overlays. In (D), three serial optical sections (inserts;1-3) of the circled area illustrate the absence of close contact between GAD terminals and a AVP cell body. Scale bar, 5μm

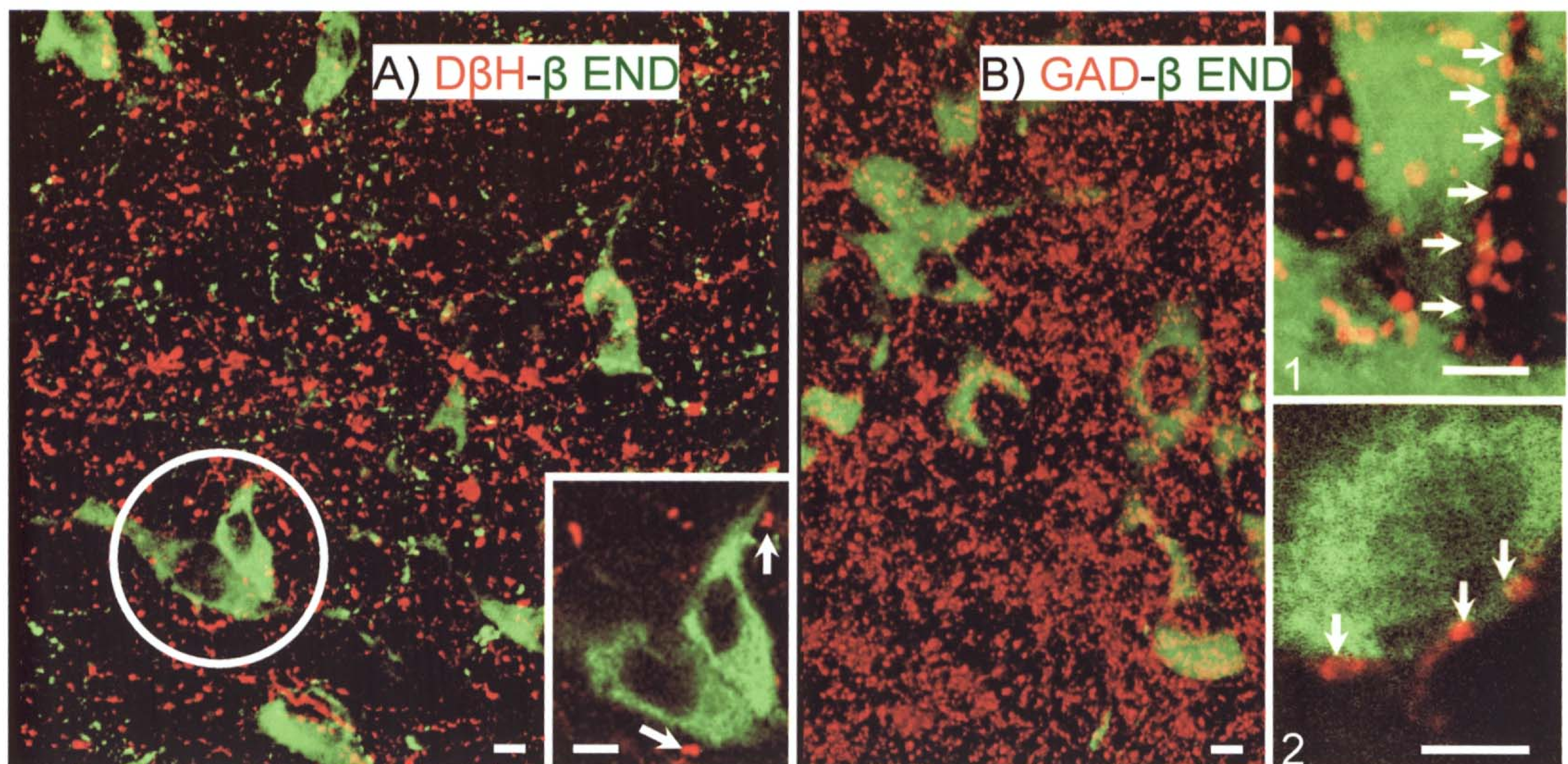


Fig 9.3 Confocal images of noradrenergic (DβH) and GABA (GAD) terminals (red), and β-endorphin cell bodies (red) in the arcuate nucleus (ARC). The stacked confocal images (A and B) show dense DβH and GAD innervation of the ARC, while close contacts were identified in a series of 0.46μm sections. From circled area in (A), the example of a single optical slice (insert) shows DβH terminals near to β-endorphin cell bodies but not in close contact (arrows). In (B), the enlarged confocal projection (1) illustrates a GAD fibre (red, indicated by arrows) moving towards the β-endorphin cell (green), and in close contact at the end. A single optical slice (2) shows the close contact (arrows) of GAD terminals with a β-endorphin cell body. Scale bar, 5μm

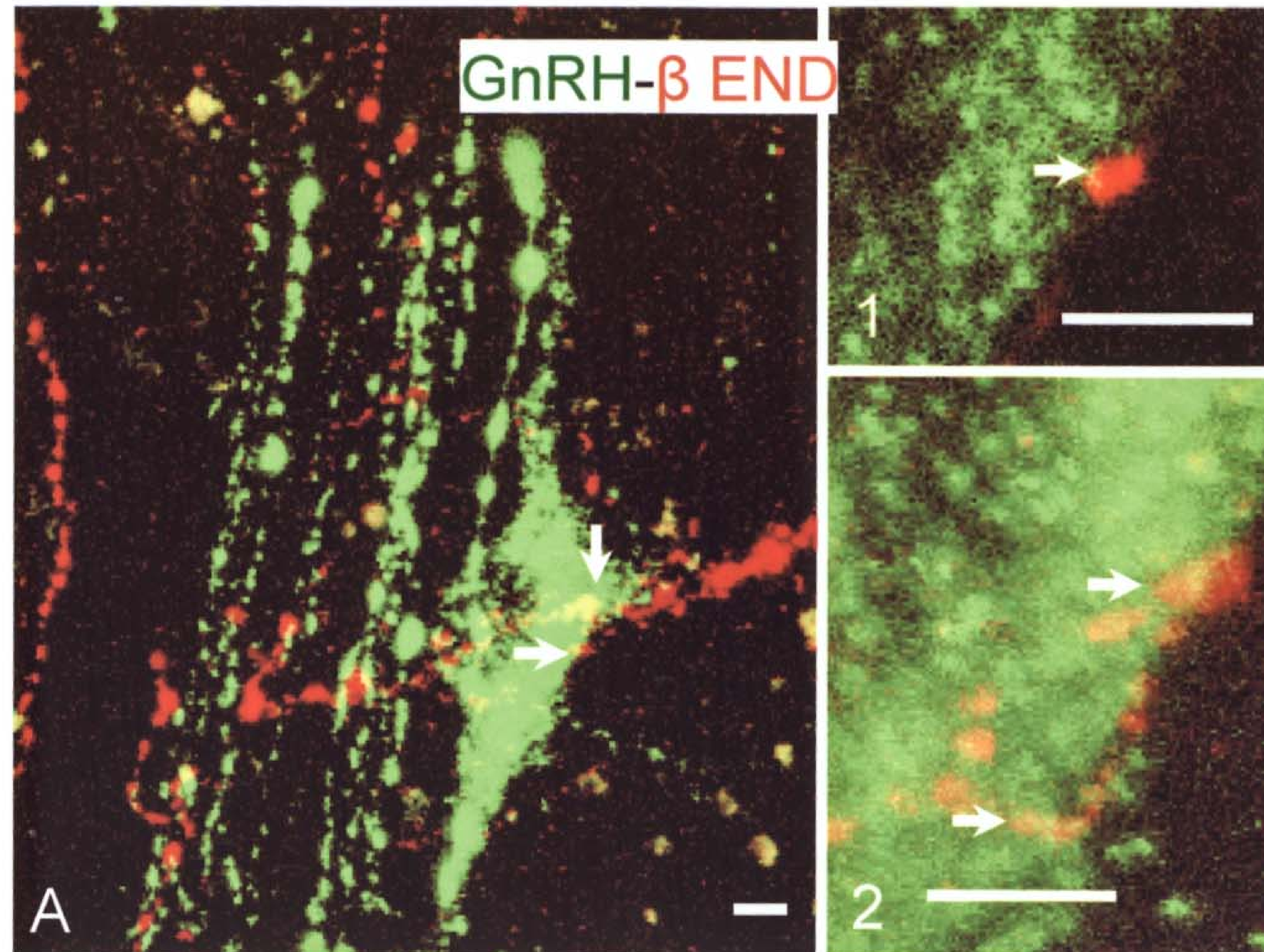


Fig 9.4 A rare example of β-endorphin terminals (red) in close contact with a GnRH cell body (green) in the mPOA. Arrows indicate close contacts (yellow). The main figure (A) is a stack of all optical slices containing this neurone, while the right panels show a single optical section (1) and an enlarged confocal projection (2). In (A), two close arrows draw attention to close contacts between GnRH and β-endorphin fibres. Scale bar, 5μm

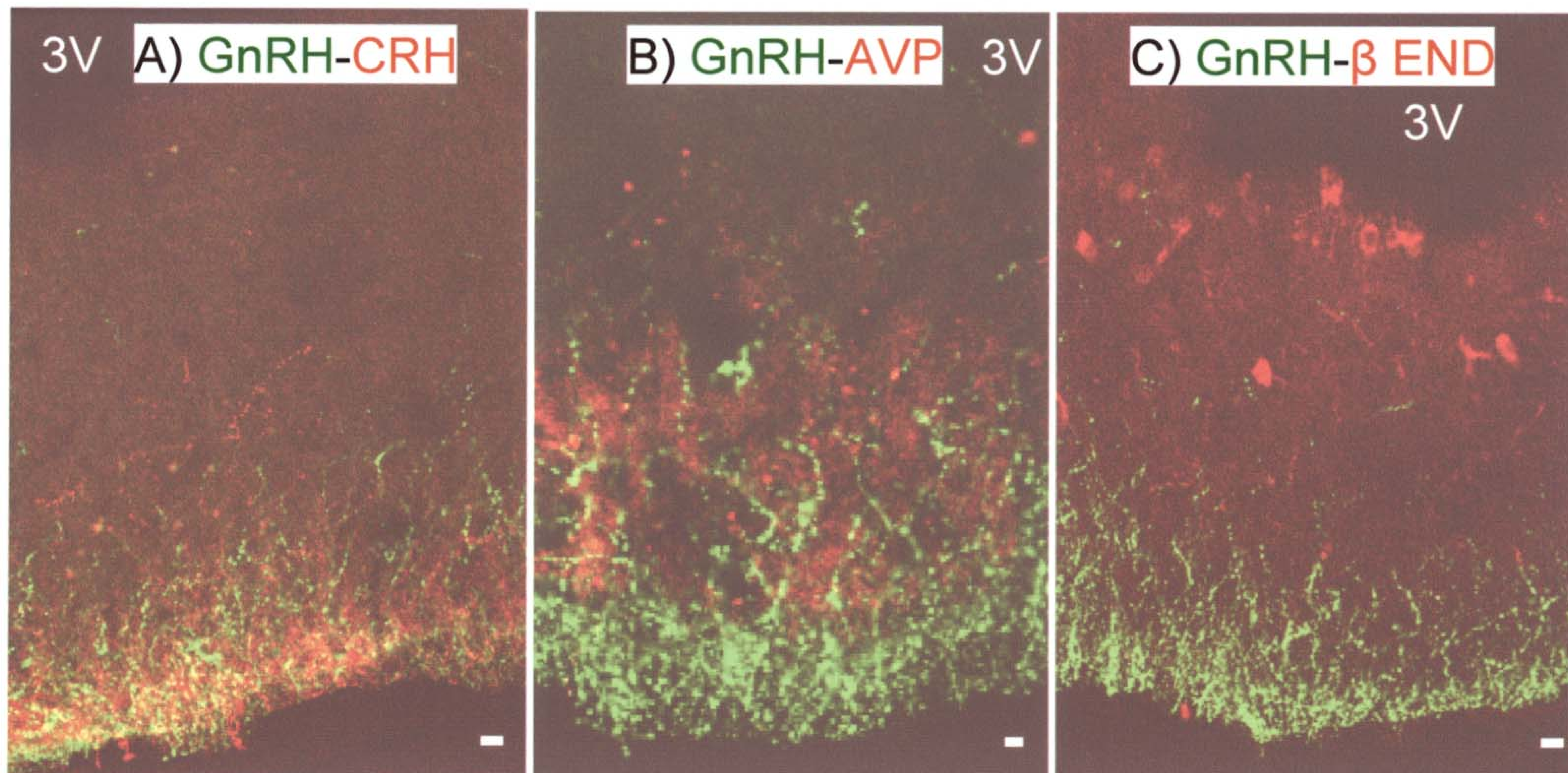


Fig 9.5 Three representative single plane confocal images of the median eminence (ME) showing GnRH (green) and CRH(A), AVP (B) or β-endorphin (C) terminals (red). Abundant CRH terminals are with GnRH terminals (yellow) in the external zone of ME (A), however no interaction between AVP (B) or β-endorphin (C) and GnRH terminals is apparent. Few β-endorphin cell bodies (C) are present in the internal zone of ME. 3V: third ventricle. Scale bar, 5μm

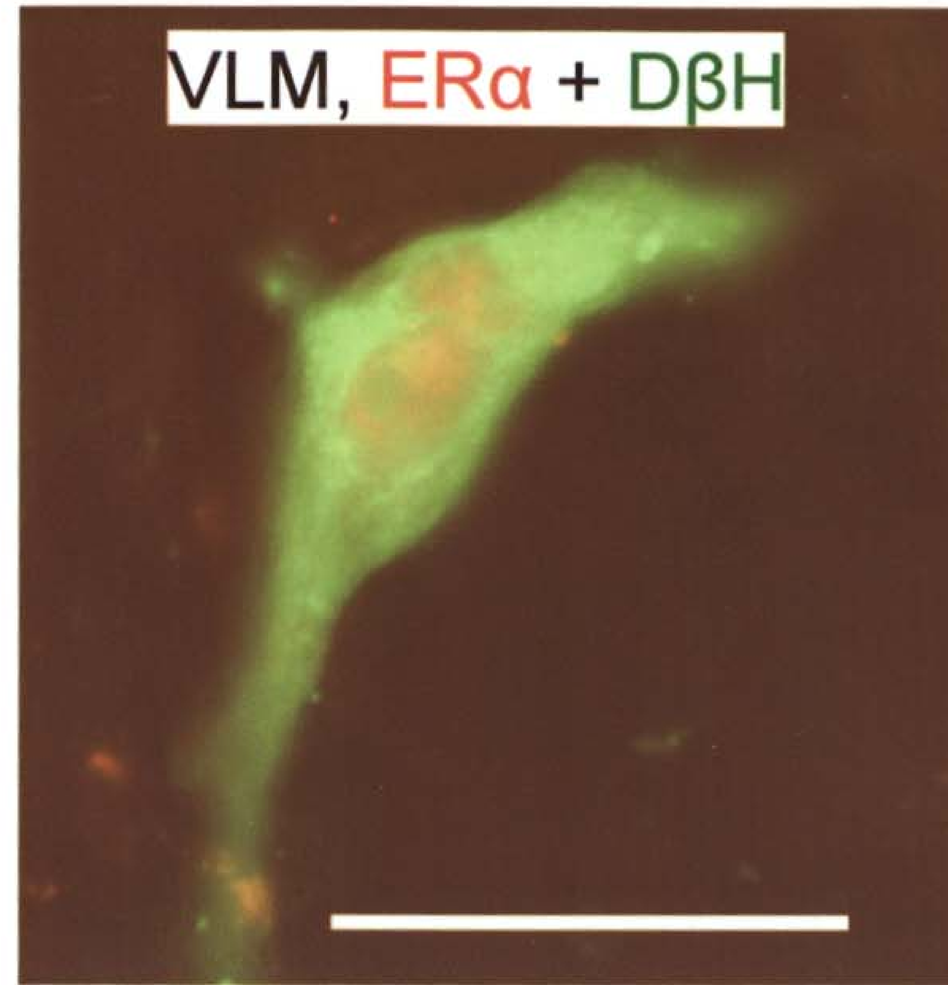


Fig. 9.6 A representative image of the caudal brainstem (ventrolateral medulla, VLM) showing a ERα (red nucleus) containing noradrenergic (DβH; green) neurone. Scale bar 50μm

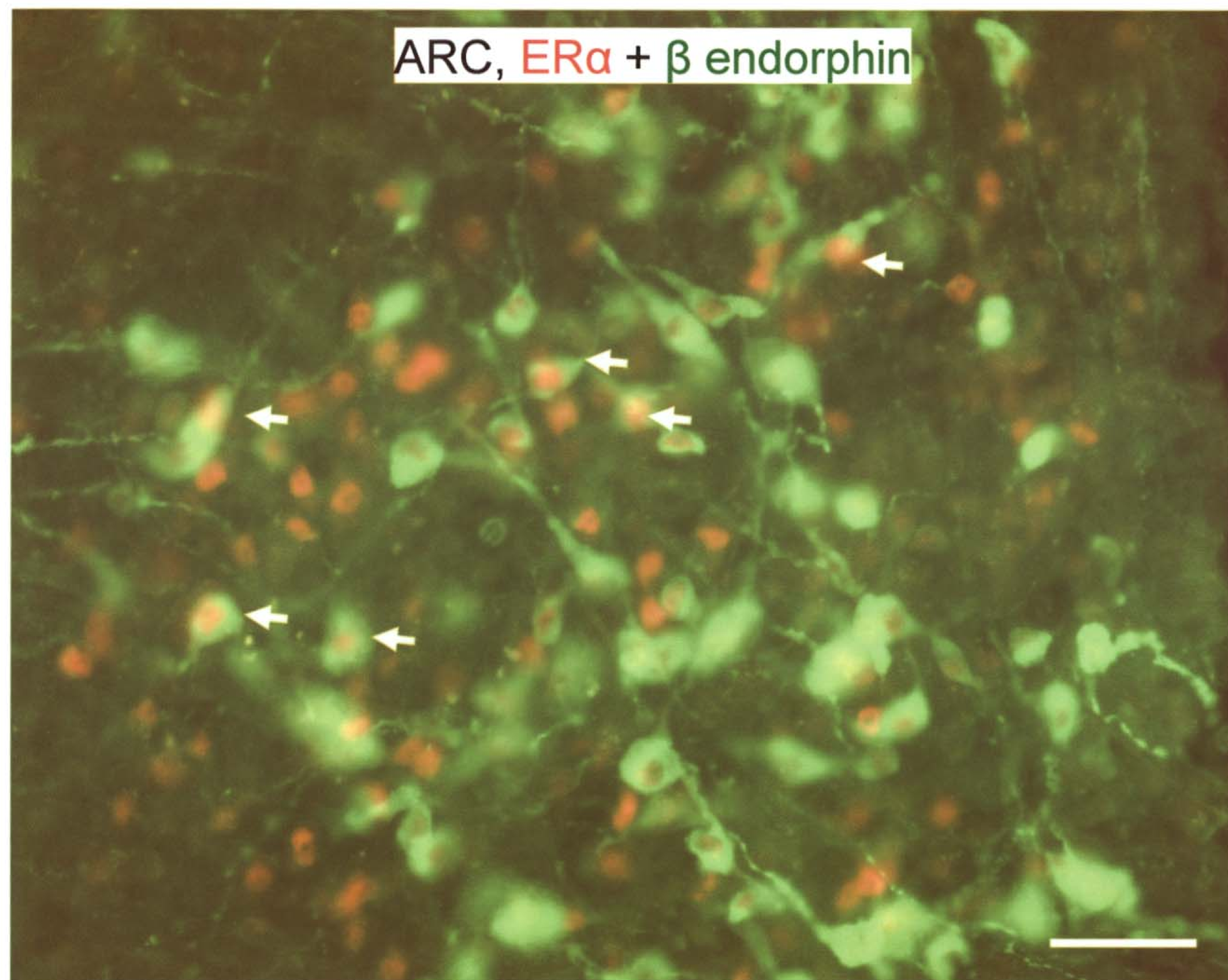


Fig. 9.7 A representative image of the arcuate nucleus (ARC) showing ERα (red nucleus) containing β-endorphin neurones (green; arrows). Scale bar 50μm

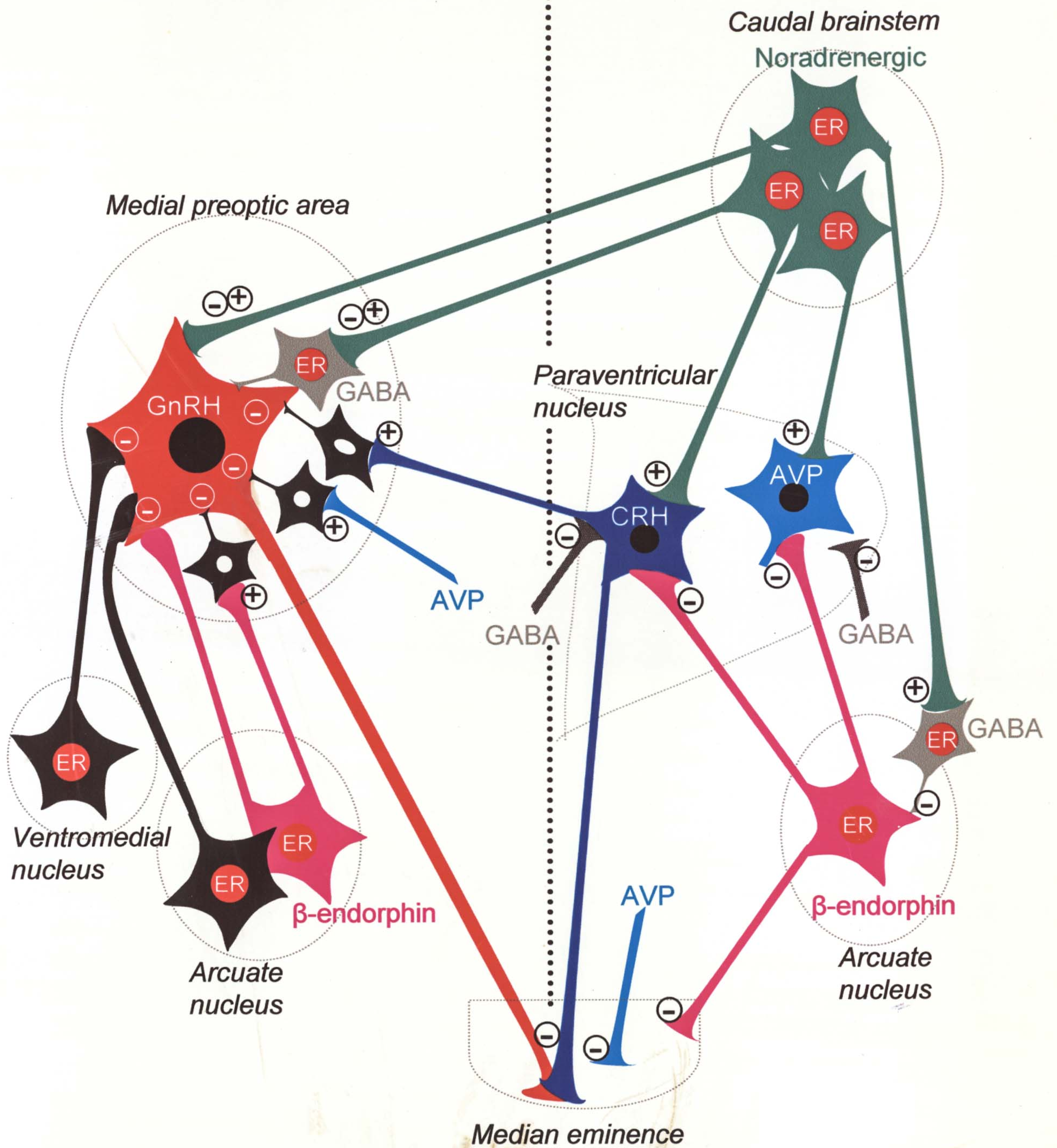


Fig 9.8 Schematic representation of central circuitry by which stress and reproduction interact. Black neurones are of unknown phenotype

Chapter 10

Neuronal responses in the brainstem and hypothalamic nuclei following insulin treatment: delineating the mechanisms involved in the disruption of the GnRH surge in the ewe

Neuronal responses in the brainstem and hypothalamic nuclei following insulin treatment: delineating the mechanisms involved in the disruption of the GnRH surge in the ewe

ABSTRACT

The aim of the present study was to determine the central mechanism(s) involved in insulin-induced disruption of the GnRH surge at the end of the follicular phase of the oestrous cycle. Intact ewes were either given saline or insulin (5 IU/kg, i.v.) at 35 h after progesterone withdrawal and sacrificed 3 h later. There was a marked increase in the proportion of Fos-positive noradrenergic neurones in the brainstem (NTS and VLM) of insulin treated ewes. In the hypothalamic PVN, insulin treatment also increased the proportion of Fos-positive CRH neurones (from 2 to 98%) and the proportion of Fos-positive magnocellular (from 0 to 3%) and parvocellular (from 2 to 46%) AVP neurones. Interestingly, after insulin treatment, despite the increase in total Fos-positive neurones in the ARC, there was a marked reduction (from 47 to 1%) in the number or proportion of β-endorphin neurones positive for Fos. Similarly, in the ARC, ERα-positive neurones colocalising with Fos decreased after insulin treatment (from 7 to 3%). Conversely, in the VMN, ERα-positive neurones with Fos increased (from 7 to 22%) alongside an increase in the total number of Fos-positive neurones. None of the GnRH neurones was Fos-positive in either treatment group at this stage of the follicular phase.

In summary, the disruption of the GnRH surge following insulin treatment is associated with increased Fos-protein in noradrenergic neurones in the caudal brainstem, CRH and AVP neurones in the PVN and by contrast suppression in β-endorphin neurones of the ARC. However, there is an increase in Fos in ARC neurones that are neither β-endorphin nor ERα-positive. Furthermore, ERα-positive neurones with Fos increased in the VMN as a result of insulin treatment. Overall, a complex system of neurones in both the brainstem and hypothalamus are implicated in the stress-induced suppression of the GnRH surge.

INTRODUCTION

Normal reproductive function is disrupted by insulin treatment via a suppression of gonadotrophin releasing hormone-luteinising hormone (GnRH-LH) release in the hypothalamus of both male and female sheep (Clarke *et al.*, 1990; Adam & Findlay, 1998; Medina *et al.*, 1998). Insulin-induced hypoglycaemia is also associated with increased release of hypothalamic corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal blood to increase pituitary release of ACTH in the ewe (Smith *et al.*, 2003a). From a mechanistic point of view, glucose responsive neurones have been identified in the area postrema (AP), a region in the caudal brainstem outside a blood-brain barrier that has also been associated with disruption of reproductive function (Schneider & Zhu, 1994; Adachi *et al.*, 1995; Murahashi *et al.*, 1996). The AP has neural and vascular connections with the nucleus of solitary tract (NTS) and the ventrolateral medulla (VLM) (Roth & Yamamoto, 1968). The latter are the two main areas containing noradrenergic neurones in the caudal brainstem that send projections to the paraventricular nucleus (PVN) to synapse with CRH/AVP neurones (Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988; Michaloudi *et al.*, 1997; Scott *et al.*, 1998). However, it is not known whether these noradrenergic neurones are associated with activation of the PVN and disruption of GnRH secretion in the ewe.

Indeed, controversy exists about the participation of the brainstem in suppression of LH release in sheep during sudden decreased glucose availability. In one study, two hours after a low dose of insulin, *c-fos* mRNA was not detected in any area of the brainstem, but *c-fos* and CRH mRNA did increase in the PVN (Adam & Findlay, 1998). Others did detect Fos-protein in the AP in response to a higher dose of insulin; and ablation of the AP prevented the inhibition of LH release induced by decreased glucose (Vellucci & Parrott, 1994; Ohkura *et al.*, 2000). However, confusingly, intracerebroventricular administration of CRH does not decrease LH release in steroid-treated ovariectomized (OVX) ewes or castrated rams (Clarke *et al.*, 1990; Caraty *et al.*, 1997).

The relative importance of AVP and CRH during stress responses is unclear (Dobson & Smith, 2000). A role for AVP mediating insulin-induced inhibition of LH remains elusive in the ewe and controversy also exists in other species (Clarke *et al.*, 1990; Heisler *et al.*, 1994; Chen *et al.*, 1996; Cates *et al.*, 1999). Furthermore, other compounds such as opioids may be involved in LH suppression after insulin or 2-deoxy-D-glucose (2DG) treatment in the ewe (Clarke *et al.*, 1990; Dobson & Smith, 2000). Clearly, there is no comprehensive picture of events in the ewe brain following sudden decreased glucose leading to suppression of GnRH-LH (surge) release. It is worth clarifying here that following insulin, the induced hypoglycemia, and not insulin itself, is responsible for LH suppression (Clarke *et al.*, 1990).

In order to increase understanding of normal GnRH release, an OVX ewe model has been developed in which oestradiol (E_2) induction of the GnRH surge has been divided into three stages: first, activation of E_2-sensitive neurones; second, transmission of a message to GnRH neurones through E_2-responsive interneurones(s); and a final stage involving initiation of GnRH surge release (Evans *et al.*, 1997). Various stressors, e.g. transport, endotoxin administration or insulin-induced hypoglycemia have suppressive effects on different stages of this GnRH surge-induction process (Smith *et al.*, 2003b).

We have recently proposed that several neuronal pathways are involved in control of normal GnRH release and during stress-induced suppression (Dobson *et al.*, 2003). The present study aims to verify some of these hypotheses. Following insulin administration during the transmission stage of a spontaneous follicular phase, we examined alterations in the activity of brainstem noradrenergic neurones and hypothalamic neurones containing CRH, AVP, β-endorphin and ERα. Activation of these cell types was verified using immunohistochemical detection of Fos-protein.

MATERIALS AND METHODS

Animals, treatments and blood sample or brain tissue collection

During the breeding season, 12 mature ewes were penned indoors for at least one week before the experiment. During this period, the animals were acclimatized to

the environment and to human contact to minimize the impact of handling and collecting blood samples. Ewes were fed hay *ad libitum* and had free access to water. All procedures were conducted under UK Home Office regulations for experimentation on living animals (License number: PPL 40/2681).

To synchronize the follicular phase, two intravaginal progesterone-releasing devices (Controlled Internal Drug Release; InterAg, Hamilton, New Zealand) were placed for 7 days into each of the ewes to establish circulating luteal phase progesterone concentrations. At the time of progesterone withdrawal, a synthetic prostaglandin (Lutalyse, Schering-Plough, Harefield, UK) was given by i.m. injection to ensure that all corpora lutea had regressed. To facilitate repeated blood collection, two days before the experiment, an indwelling catheter (Medical grade silastic tubing, internal diameter-1.01 mm, VWR) was inserted into the jugular vein of all ewes under local anesthesia and patency was maintained with heparinized saline (100 IU/ml).

Ewes received either insulin (n=6; 5 IU/kg) or saline (n=6) via the jugular catheter 35 h after progesterone withdrawal. Hourly blood samples were collected from 34 h after progesterone withdrawal to the time of sacrifice. The samples were collected into heparinized tubes and immediately centrifuged at 1000g for 20 min. Plasma was stored at -15°C until analyzed. Three hours after treatment, ewes were sacrificed and heads were perfused as described in chapter 2. To aid rapid dissection and fixation, treatment of ewes was staged so pairs of animals were sacrificed every 2 hours.

Hormone estimation

LH, cortisol and glucose concentrations were determined as described previously (chapter 2). Intra-assay coefficients of variation for cortisol and glucose were 8.4 and 1.7%, respectively. Both inter- and intra-assay coefficients of variation for LH assay were <9.0%. Sensitivity of the cortisol and LH assay was 0.4 and 0.3 ng/ml, respectively.

Hormone data statistical analysis

Data were expressed as mean ± SEM. LH, cortisol and glucose data were log_{10} transformed before statistical analysis to achieve homogeneity of variance. Mean plasma cortisol and glucose concentrations in all ewes before and after insulin administration and between treatments were compared by generalized linear model (GLM ANOVA) procedures and Tukey's pairwise comparison. Probabilities of less than 0.05 were considered significant. Statistical analyses were performed using Minitab release 13.20 statistical software (Minitab Inc., State College, PA, USA).

Immunohistochemical procedures

Double-labelled (Fos with DβH or CRH or AVP or β-endorphin or ERα or GnRH) immunohistochemistry was performed using free-floating sections as described in Chapter 2. Details of the secondary antisera used in various combinations with primary antisera are described in Table 10.1.

Cell count and statistical analysis

1) Fos with DβH: In the NTS and VLM (6 sections/ewe at 1.0 mm interval, Fig 10.2 A-F), all single or double-labelled noradrenergic (DβH) neurones were counted unilaterally per section. Due to the high density of noradrenergic neurones in the locus coeruleus (LC; 3 sections/ewe at 1.0 mm interval, Fig 10.2 G-I), a minimum of 50 neurones per section were randomly examined for double-labelling.

2) Fos with CRH or AVP: Observations were made from sets of pictures (2-3 fields per section, covering the unilateral PVN; 6 sections/ewe/combination at 280 μm interval, Fig 10.4) taken with x10 microscopic power. Single- or double-labelled magnocellular and parvocellular AVP neurones were counted separately based on the cell size criteria (magnocellular, 30 x 25 μm; parvocellular, 25 x 5 μm; Fig 10.6, insert 1 and 2) as described previously for goats (Kikusui *et al.*, 1997).

3) Fos with β-endorphin: Single- or double-labelled neurones were counted over the unilateral ARC for every section (2 sections/ewe at 400 μm interval, Fig 10.7).

4) Fos with ERα: For single-label Fos or ERα-positive neurones, counting was carried out over the unilateral mPOA, ARC and ventromedial nucleus (VMN; per ewe 2 sections of mPOA at the organum vasculosum of lamina terminalis at 400 μm interval and 3 sections of VMN/ARC at 400 μm interval, Fig 10.7). For double-label counting, a minimum of 50 Fos and ERα-positive neurones were checked randomly per area per section.

5) Fos with GnRH: Single- or double-labelled GnRH neurones were counted in 18 sections/ewe at 400 μm intervals through the hypothalamus starting from diagonal band of broca (dBB) to the mammillary bodies (MB).

Irrespective of the total number of each neurone type counted in each section/ewe, the proportion in a section that was Fos-positive was consistent, therefore, for each combination, the total number of single- or double-labelled neurones counted from each section was pooled for each area in each ewe brain. Data were expressed as mean total labelled neurones (±SEM) counted per area per ewe and as mean percentage (±SEM) of total number of single and double-labelled neurones. Data were $\log_{10}$ transformed before statistical analysis to achieve homogeneity of variance. Data values between treatments were compared by GLM ANOVA procedures and Tukey's pairwise comparison. Probabilities of less than 0.05 were considered significant.

RESULTS

Glucose, cortisol and LH responses

Administration of insulin resulted in a decrease in circulating plasma glucose that remained low until sacrifice (Fig 10.1). Plasma cortisol concentrations were higher after insulin treatment (Fig 10.1). No LH surges were observed (concentrations <0.6 ng/ml) except in one ewe after saline treatment in which LH was >10 ng/ml for >5 hours before sacrifice.

Immunohistochemistry

Combinations of several immunohistochemical investigations were carried out on brain tissue from all the ewes, except for the one that had an LH surge at the time of sacrifice (saline-treated) for which only Fos/GnRH staining was determined.

Brainstem: Insulin treatment increased the number of Fos-positive neurones in the NTS, VLM and LC regions compared to saline treated ewes (Table 10.2, Fig 10.2 A-I). Also, in the AP (Fig 10.2E-F, Fig 10.3A-B), there were more Fos-positive neurones after insulin treatment (164±48 *versus* 0.5±0.3 neurones), as well as in the dorsal tegmental nucleus (DTg) and the lateral recess of 4th ventricle above the LC (Fig 10.2G-I, Fig 10.3E-F).

In controls, noradrenergic neurones were not Fos-positive, however in the insulin treated animals, in the VLM 53% noradrenergic neurones express Fos and in the NTS and LC, 20% noradrenergic neurones express Fos ~50-60% Fos-positive neurones in the VLM and LC regions were noradrenergic (Table 10.2; Fig 10.3C-D). There was considerable inter-animal variation in the number of noradrenergic neurones positive for Fos in the LC.

PVN: A marked increase in Fos-positive neurones was observed after insulin treatment (Table 10.3). The majority of this Fos occurred in CRH neurones (Table 10.3, Fig 10.4A-C, Fig 10.5). Insulin treatment also increased the proportion of magnocellular (3%) and parvocellular (46%) AVP neurones positive for Fos (Table 10.4, Fig 10.4D-F, Fig 10.6). It should be noted that only 4-5% of the AVP neurones were parvocellular out of ~1200 counted in the PVN.

ARC: A substantial number (>100) of Fos-positive neurones were present in the ARC of saline treated ewes (Table 10.5, Fig 10.7, Fig 10.8), and ~70% contained β-endorphin. However, only ~50% of the β-endorphin neurones were positive for Fos. Interestingly, although the number of Fos-positive neurones increased in the ARC after insulin, the proportion of β-endorphin neurones positive for Fos reduced dramatically to 1%.

ARC, VMN and mPOA: Between treatments, there was no difference in the total ERα-positive neurones in the ARC, VMN or the mPOA (Table 10.6).

In the ARC, VMN and mPOA of the saline treated ewes, there were many Fos-positive neurones. In the ARC and VMN, the total number of Fos-positive neurones increased after insulin treatment. In the ARC, the percentages of both Fos-positive neurones with ERα, and ERα-positive neurones with Fos, decreased after insulin (image not shown). In contrast, in the VMN, ERα-positive neurones with Fos increased after insulin treatment (Table 10.6, Fig 10.9).

Hypothalamus (from diagonal band of broca to mammillary bodies): No Fos-immunoreactivity was found in GnRH neurones throughout the hypothalamus in all the saline and insulin treated ewes, with the exception of the one ewe that had an LH surge at the time of sacrifice after saline treatment, in which 57% (30 out of 52 GnRH neurones, Fig 10.10) GnRH neurones were Fos-positive.

DISCUSSION

Mapping of Fos-protein following insulin treatment suggested *c-fos* gene activation in noradrenergic neurones in the caudal brainstem, CRH and AVP neurones in the PVN and by contrast suppression in β-endorphin neurones in the ARC. In the ARC and VMN, insulin treatment respectively suppressed and activated *c-fos* expression in ERα-positive neurones. This suggests the involvement of both the brainstem and hypothalamus in insulin-induced disruption of GnRH release.

Increase in Fos-immunoreactivity in noradrenergic neurones of the brainstem

This is the first detailed study of the ewe brainstem establishing that insulin increases Fos-protein in the AP, NTS, VLM and LC regions of the brainstem, and suggests *c-fos* gene activation in approximately 50% noradrenergic neurones in the NTS and VLM. One previous brief study in sheep reported an increase in Fos-positive neurones in the AP while another study was unable to find *c-fos* gene expression

throughout the brainstem after a lower dose (0.5 IU/kg) of insulin, possibly due to the sensitivity of the *in situ* hybridization method (Vellucci & Parrott, 1994; Adam & Findlay, 1998). Noradrenergic neurone populations in the NTS, VLM and LC of the ewe brainstem have been mapped previously (Scott *et al.*, 1999), but not with regard to stress responses. The increase in AP activity observed in the present study after insulin could be related to the disruption of GnRH release as removal of the AP prevents the inhibitory effects of decreased glucose availability on LH release in the rat (Cates & O'Byrne, 2000).

Although half the Fos-positive neurones were not noradrenergic in the VLM and LC, a substantial proportion (53%) of noradrenergic neurones were Fos-positive after insulin in the VLM region probably stimulated by projections from glucose responsive AP neurones as in the rat (Cunningham *et al.*, 1994; Adachi *et al.*, 1995). This concurs with evidence that low glucose increases release of noradrenaline in the PVN and leading to suppression of LH release, and this is reversed by administration of a catecholamine synthesis inhibitor directly into the PVN (Nagatani *et al.*, 1996). Furthermore, immunotoxic destruction of NTS and VLM noradrenergic neurones prevents increases in cycle length in response to low glucose in rats (I'Anson *et al.*, 2003). However, after this immunodestruction of PVN-projecting noradrenergic neurones, normal oestrous cycles were observed both in the presence and absence of adequate glucose, suggesting that noradrenergic neurones signalling hypoglycaemia are separate from those required for normal oestrous cyclicity (I'Anson *et al.*, 2003).

A considerable number of non-noradrenergic neurones were Fos-positive in the NTS of insulin-treated ewes. In the rat, one subpopulation of these non-noradrenergic neurones might be the excitatory glutamate neurones that project extensively to the VLM region to synapse with neurones projecting to the PVN (Sawchenko & Swanson, 1982; Kawano & Masuko, 1996). Another subpopulation may contain glucagon-like peptide 1 which is synthesized in non-noradrenergic neurones of the NTS with terminals projecting to PVN resulting in increased ACTH release (Larsen *et al.*, 1997; Kinzig *et al.*, 2003). In addition, non-noradrenergic PVN-projecting NTS neurones may also synthesize somatostatin, substance-P and enkephalin (Sawchenko *et al.*, 1988; Sawchenko *et al.*, 1990). Of these, somatostatin and substance-P are inhibitory

but enkephalin is stimulatory to stress-induced HPA activation (Larsen *et al.*, 1993; Itoi *et al.*, 1998).

Noradrenergic neurones in the LC do not contain ERα but there is colocalisation in the NTS and VLM of the ewe (Scott *et al.*, 1998). Whether these ERα play a role to potentiate the stress axis response to insulin treatment through the observed Fos-positive neurones in the NTS and VLM remains to be determined.

Although the present study was not designed to resolve all events leading to normal GnRH surge release, there was no Fos-immunoreactivity in brainstem noradrenergic neurones 38 h following progesterone withdrawal. In the OVX ewe after a surge-inducing signal, Fos-immunoreactivity in brainstem noradrenergic neurones occurs within 2 h after E_2 administration followed by delayed release (16 h) of noradrenaline in the mPOA but Fos-was detected for only 3-4 h (Clarke *et al.*, 1999; Clarke *et al.*, 2001). Therefore it remains possible that 38 h after progesterone withdrawal, Fos responses in brainstem noradrenergic neurones may already have occurred during the putative E_2 activation phase.

Increase in Fos-immunoreactivity in CRH neurones of the PVN

Insulin treatment did not increase the number of CRH neurones in the PVN but did cause a robust increase in Fos-immunoreactivity, with 98% CRH neurones becoming Fos-positive. This pattern is consistent with an earlier study in rams where marked increases in *c-fos* and CRH gene expression in the PVN were observed after insulin treatment (Adam & Findlay, 1998). The increase in Fos-protein suggesting activation of CRH neurones concurs with the increase in CRH release in pituitary portal blood after insulin treatment (Engler *et al.*, 1989; Caraty *et al.*, 1990; Smith *et al.*, 2003a).

Considering the consequences of increased CRH neurone activation, these neurones project to the external zone of ME where CRH and GnRH fibres are closely located (chapter 9). In addition, administration of CRH to the ME or CRH antagonist to the PVN of fasted rats, respectively suppressed or restored LH release (Maeda *et al.*, 1994; Ortega *et al.*, 1994; Frias *et al.*, 1997).

Increase in Fos-immunoreactivity in AVP neurones of the PVN

The present study is the first to show a marked increase in both magnocellular (3% of 1200 neurones) and parvocellular (46% of 60 neurones) Fos-positive AVP neurones after insulin treatment. An increase in AVP release into portal blood follows (Engler *et al.*, 1989; Caraty *et al.*, 1990; Smith *et al.*, 2003a). Considering the small population of parvocellular AVP neurones in the PVN, it is intriguing whether this marked increase in AVP in portal blood comes from only parvocellular AVP neurones, or do magnocellular AVP neurones also contribute? Parvocellular AVP neurones are the primary source of input to the anterior pituitary, although studies in the rat suggest that magnocellular AVP neurones, as well as projecting directly to the posterior pituitary are also capable of releasing AVP from axons in the median eminence (Holmes *et al.*, 1986; Buma & Nieuwenhuys, 1987; Antoni *et al.*, 1990; Wotjak *et al.*, 2002).

There is potential for noradrenaline to stimulate AVP neurones following insulin treatment as more than 80% AVP neurones in the PVN receive noradrenergic terminals in the ewe (chapter 9). Also, intracerebroventricular noradrenaline increases CRH and AVP release in both the rat and ewe (Plotsky *et al.*, 1989; Liu *et al.*, 1991). Furthermore, increased noradrenaline in the PVN stimulates CRH and AVP transcription in rat (Itoi *et al.*, 1999; Haisenleder, 2000; Cole & Sawchenko, 2002).

The absence of effects after intracerebroventricular administration of AVP in unstressed sheep, or AVP antagonist in insulin-treated rats, suggest that AVP is not primarily involved in suppression of LH secretion (Clarke *et al.*, 1990; Cates *et al.*, 1999). Also, there was no interaction of AVP and GnRH fibres in the ME (chapter 9). However, in ewe, endotoxin-induced suppression of GnRH release coincident with AVP and CRH release suggests the involvement of these peptides (Battaglia *et al.*, 1998). Whether the observed increases in Fos responses in parvocellular and magnocellular AVP neurones in response to insulin have any role in disruption of GnRH release needs to be further investigated.

As there are markedly more Fos-positive CRH neurones than AVP neurones, the relative role of AVP and CRH in suppressing GnRH release needs clarification. In the ewe, AVP is thought more important than CRH in inducing the release of ACTH

from pituitary (Smith *et al.*, 2003a). In contrast to our Fos-positive data for CRH and AVP neurones after insulin, the molar ratio of CRH/AVP changes markedly in favour of AVP in this species (Engler *et al.*, 1989). It is intriguing to consider the greater release of AVP compared to CRH in view of our observations.

There may be another role for AVP in potentiating the effects of CRH on HPA activity in sheep (McFarlane *et al.*, 1995). In the rat, the average number of neurones in the PVN that contained AVP and CRH colocalisation (~33) constitutes only about 3% of the total number of AVP neurones in PVN (Sawchenko *et al.*, 1984). After insulin, there were twice as many CRH and AVP colocalising cell bodies suggesting that acute stress induces AVP gene transcription in a subpopulation of CRH synthesizing neurones (Paulmyer-Lacroix *et al.*, 1994). Overall, it remains to be established whether the Fos-positive AVP population has a role in potentiating the effects of CRH through colocalisation in CRH cell bodies, or AVP in its own right is able to suppress GnRH.

In the present study after saline, it is clear that 2% CRH neurones colocalised with Fos, possibly reflecting tonic activation. In addition, after insulin stimulation there is quite a high proportion (>20%) of cells containing Fos but not CRH or AVP indicating involvement of other cell types. Our earlier studies (chapter 9) would suggest that a proportion of these may be principally γ-amino butyric acid (GABA).

Decrease in Fos-immunoreactivity in β-endorphin neurones of the ARC

Following insulin treatment, the marked decrease (from 47 to 1%) in Fos-positive β-endorphin neurones suggests suppression of *c-fos* gene activation in β-endorphin neurones. This implies that insulin treatment restrains the activity of β-endorphin neurones. Whether this is related to the removal of inhibitory opioid tone which plays an important role in preventing premature activation of GnRH neurones near the end of the follicular phase of the oestrous cycle (Domanski *et al.*, 1991; Dobson *et al.*, 2003), or whether this is associated with removal of the inhibitory role of β-endorphins in the hypothalamus on the HPA axis, needs further investigation. Indeed, a pathway to exert the latter effect exists as β-endorphin neurones from the ARC project to the PVN in the rat (Baker & Herkenham, 1995). Functionally, it is

well known that opioids suppress CRH release and thus removal of opiates evokes CRH mRNA as well as CRH and AVP release *in vivo* (Plotsky, 1986; Lightman & Young, 1988; Alexander & Irvine, 1995; Hockings *et al.*, 1995; Janssens *et al.*, 1995). Furthermore, naloxone, an opioid antagonist, increases basal CRH release and reverses β-endorphin-induced suppression of CRH release from rat hypothalami *in vitro* (Yajima *et al.*, 1986; Tsagarakis *et al.*, 1990).

But by what mechanism(s) does insulin treatment suppress β-endorphin tone? This could be an important role for GABA as around 60% β-endorphin neurones in the ARC receive input from GABA terminals in the ewe (chapter 9) although the origin of this GABA input is not clear. However, following insulin treatment in rats, GABA turnover increased in the VMN (Beverly *et al.*, 2001). Thus, there may be interplay between GABA, β-endorphin and CRH neurones following insulin treatment, by which increased GABA inhibits β-endorphin suppressive tone thus CRH neurones in PVN are free to secrete CRH. This is in line with the present results; after insulin, there were fewer Fos-positive β-endorphin neurones in the ARC simultaneously with increased *c-fos* gene activation in CRH neurones in the PVN.

A conundrum remains, however, as this suppression of β-endorphin activity following insulin treatment does not concur with previous studies in the ewe in which non-specific opioid antagonists reverse insulin-induced LH suppression (Clarke *et al.*, 1990; Dooley *et al.*, 1998; Dobson & Smith, 2000). One possibility could be that other opioids such as enkephalin and dynorphin might be involved, a network for which exists throughout the ovine hypothalamus (Marson *et al.*, 1987; Walsh *et al.*, 2001). Among the opioids, enkephalin has stimulatory effects while β-endorphin and dynorphin inhibit CRH neurones (Itoi *et al.*, 1998).

An important finding in the controls, in relation to the normal GnRH surge generating mechanisms, is the presence of Fos in ~50% β-endorphin neurones. Ewes in the present study were sacrificed in the mid to late follicular phase of the oestrous cycle, corresponding to the time when inhibitory opioid tone plays an important role in preventing premature activation of GnRH neurones (Dobson *et al.*, 2003). During the follicular phase of the ewe oestrous cycle, there is a two-fold increase in opioid receptors as well as an increase in β-endorphin concentrations in the median eminence (Domanski

et al., 1991; Conover *et al.*, 1993). But how β-endorphin neurones exert this effect on GnRH neurones is another question that remains to be answered. Stimulatory noradrenergic, glutamate, nitric oxide and/or inhibitory GABA neurones may be potential mediators as opioids restrain this stimulatory input and accelerate inhibitory input to GnRH neurones in the rat (Kalra, 1993; Yilmaz *et al.*, 1996; Bhat *et al.*, 1998; Faletti *et al.*, 1999). Also, in the ewe, an opioid antagonist suppresses preoptic GABA, suggesting opiate action on GABA neurones but no concurrent change in preoptic noradrenaline release questions opiate-noradrenaline interactions (Robinson & Kendrick, 1992a).

Decrease in Fos-immunoreactivity in ERα-positive neurones of the ARC

This decrease may be partly due to the decrease in Fos-positive β-endorphin neurones after insulin treatment observed in the present study. In the ARC, ERα-containing neurones are positive for β-endorphin (32%) and others peptides, such as neuropeptide-Y (NPY, 10%), glutamate (60%) and neurokinin-B (97%) (chapter 9) (Lehman & Karsch, 1993; Skinner & Herbison, 1997; Goubillon *et al.*, 2000; Pompolo *et al.*, 2003b). How the activity of these ERα-positive neurones is altered following insulin treatment and whether they have a role in surge suppression needs investigation. With regard to the LH surge in the ewe, NPY is inhibitory while glutamate is stimulatory, but the involvement of neurokinin-B is not known (Caraty *et al.*, 1998b; Grindrod *et al.*, 2002; Estrada *et al.*, 2003).

However, despite the decrease in the number of ERα-positive neurones with Fos, the total number of Fos-positive neurones in the ARC increased following insulin treatment. This suggests increased activity of neurones without ERα could be involved in disruption of GnRH release. As only a few NPY neurones have ERα in the ARC, ERα-negative NPY neurones may be activated following insulin. In underfed goats, NPY neurones in the ARC inhibit the GnRH release regardless of the E_2 milieu (Ichimaru *et al.*, 2001). Furthermore, NPY is a major stimulant for HPA activity and these neurones, originating from the ARC, synapse with CRH neurones in the PVN (Liu *et al.*, 1991; Li *et al.*, 2000).

Increase in Fos-immunoreactivity in ERα-positive neurones of the VMN

The insulin-induced increase in total Fos-positive neurones, as well as the increase in ERα-positive neurones with Fos, could be involved in the disruption of GnRH release. One responsible candidate neurotransmitter could be GABA. Both E_2 and insulin increase GABA turnover in the VMN in rats (Luine *et al.*, 1997; Beverly *et al.*, 2001). It remains to be established whether ERα-positive GABA neurones are present in the ovine VMN and whether these neurones are a source of input to β-endorphin neurones in the ARC, a connection that may be involved in the regulation of HPA axis activity.

In ewes, the VMN is regarded as the primary site of E_2 action for inducing the GnRH surge (Caraty *et al.*, 1998a). This is further supported by the presence of 82% Fos-positive neurones with ERα in the controls that would have progressed to a normal GnRH surge. The main excitatory ERα-positive populations in the ewe VMN are glutamate and somatostatin (Unsworth & Robinson, 2002; Pompolo *et al.*, 2003b). Whether the activity of these neurones is altered following insulin remains an enigma.

No alteration in Fos-immunoreactivity in ERα-positive neurones of the mPOA

GnRH neurones in the mPOA do not possess ERα (Lehman & Karsch, 1993), therefore, the Fos-positive neurones in the mPOA of saline-treated controls might be the interneurones involved the GnRH surge generation process.

To initiate a GnRH surge in the ewe, glutamate neurones comprise the main excitatory population in the POA which colocalise ERα (40%) and projects terminals to GnRH neurones (Pompolo *et al.*, 2003b). The increase in GnRH following progesterone withdrawal is associated with increased glutamate tone in the POA of the E_2 treated-OVX ewes (Caraty *et al.*, 1998b). Whether the activity of glutamate neurones in the mPOA is suppressed following insulin remains to be determined. In the rat, CGRP is another population of ERα-colocalising neurones in the mPOA that are inhibitory to the GnRH surge following insulin treatment, but in the ewe, there are very few CGRP neurones present in the POA and their role in GnRH surge generation is not known (Herbison & Theodosis, 1992; Herbison *et al.*, 1993a; Li *et al.*, 2004).

Furthermore, a subpopulation (40%) of GABA neurones express ERα in the mPOA and GnRH neurones receive inputs from GABA neurones in this area (Herbison *et al.*, 1993b; Pompolo *et al.*, 2003b). Whether the activity of GABA neurones with or without ERα increases following insulin needs investigating.

Fos-immunoreactivity of GnRH neurones throughout the hypothalamus

In the present study, GnRH neurones were Fos-negative throughout the hypothalamus both in insulin and saline treated ewes, except in one saline treated ewe that was experiencing an LH surge. This suggests that Fos activation in GnRH neurones only occurs actually during the surge phase.

The presence of a substantial proportion of Fos and ERα colocalising neurones in the ARC, VMN and mPOA of the saline treated ewes suggests that the E_2-signal for GnRH surge-induction had already been delivered. In intact ewes, precise demarcation of the three stages of the follicular phase (Evans *et al.*, 1997) is not known, although our observations of Fos colocalising with GnRH or ERα-positive neurones, suggests that 38 h after progesterone withdrawal, these ewes were in the transmission phase of the surge-induction process. Therefore, insulin treatment at this time is interfering with E_2-sensitive interneurones transmitting the surge-inducing E_2-signal to GnRH neurones.

No alteration in ERα-positive neurones of the ARC, VMN, mPOA or PVN

There was no difference in the total number of ERα neurones in the ARC, VMN or mPOA after insulin. Some previous studies in rats have suggested an increase in the number of ERα neurones in the PVN and caudal brainstem after fasting or glucoprivation stress (Estacio *et al.*, 1996; Reyes *et al.*, 2001). However, we were unable to confirm this in the ewe PVN (data not shown) and were unable to establish changes in the brainstem as the intensity of ERα staining was not optimal for quantification in the brainstem.

Conclusions

Mapping of Fos-protein following insulin treatment suggests activation of *c-fos* gene expression in noradrenergic neurones in the caudal brainstem and CRH and AVP neurones in the PVN. While the role for the caudal brainstem in the regulation of PVN neurones following decreased glucose availability is well established in rats, we have confirmed similar mechanisms in ewes. The suppression of the *c-fos* in ARC β-endorphin neurones could have a potentiating influence on PVN output. In the ARC, *c-fos* expression decreases in ERα-positive neurones, in contrast to an increase in the VMN. Overall, both brainstem and hypothalamic nuclei appear to have a major role in GnRH-LH surge disruption following insulin treatment near the end of follicular phase of the oestrous cycle in ewes.

In saline-treated controls, we were unable to observe Fos-immunoreactive noradrenergic neurones in relation to the onset of the GnRH surge possible because it may have occurred earlier. The presence of Fos in the ARC β-endorphin neurones emphasised the role of these neurones to exert inhibitory tone during preovulatory period. The substantial Fos-immunoreactivity in ERα-positive neurones in the ARC, VMN and mPOA of the control ewes suggests that interneurones in these areas are important in transmitting the surge-generating E_2 signal to GnRH neurones. Collating all these observations with the presence of Fos in GnRH neurones in the ewe that was having an LH surge, we suggest that 38 h after progesterone withdrawal the ewes were in the transmission phase of the GnRH surge-generating E_2 signal.

Table 10.1 Secondary antisera used in various combinations of primary antisera

Primary antisera combination	Primary antisera (a fluorescent tag conjugated to secondary antisera)	
Fos with DβH or CRH or AVP or β-endorphin or GnRH	Fos (Anti-rabbit Cy_3)	others (Anti-rabbit FITC)
Fos with ERα	Fos (Anti-rabbit FITC)	ERα (Anti-mouse Cy_3)

Table 10.2 Mean total number (±SEM) of Fos, DβH and double-labelled neurones (Fos + DβH) in six NTS sections/ewe, six VLM sections /ewe and three LC sections/ewe after saline or insulin treatment (n=5-6 ewes per group). $p<0.05$, between treatments within an area (*)

	NTS		VLM		LC	
	Saline	Insulin	Saline	Insulin	Saline	Insulin
Fos	17±2	489±84*	1±1	109±11*	4±1	72±20*
DβH	19±3	27±4	112±5	121±5	150±0	154±2
Fos with DβH (%)	0	1±0*	0	61±5*	0	48±10*
DβH with Fos (%)	0	20±6*	0	53±2*	0	21±10#

only two out of five ewes had Fos-positive noradrenergic LC neurones.

Table 10.3 Mean total number (±SEM) of Fos, CRH and double-labelled neurones (Fos + CRH) in six PVN sections/ewe after saline or insulin treatment (n=5-6 ewes per group). p<0.05, between treatments within an area (*)

	PVN	
	Saline	Insulin
Fos	42±10	1062±38*
CRH	996±45	832±31
Fos with CRH (%)	37±8	77±2*
CRH with Fos (%)	2±1	98±1*

Table 10.4 Mean total number (±SEM) of Fos, magnocellular or parvocellular AVP and double-labelled neurones (Fos + AVP) in six PVN sections/ewe after saline or insulin treatment (n=5-6 ewes per group). p<0.05, between treatments within an area (*)

	PVN	
	Saline	Insulin
Fos	51±13	1412±101*
Magnocellular AVP	1155±48	1153±65
Fos with magnocellular AVP (%)	14±6	2±1
Magnocellular AVP with Fos (%)	0±0	3±1*
Parvocellular AVP	51±7	56±7
Fos with parvocellular AVP (%)	1±1	2±0
Parvocellular AVP with Fos (%)	2±1	46±7*

Table10.5 Mean total number (±SEM) of Fos, β-endorphin and double-labelled neurones (Fos + β-endorphin) in two ARC sections/ewe after saline and insulin treatment (n=4 ewes per group). $p<0.05$, between treatments within an area (*)

	ARC	
	Saline	Insulin
Fos	146±18	278±15*
β-endorphin	227±27	176±32
Fos with β-endorphin (%)	71±3	1±0*
β-endorphin with Fos (%)	47±8	1±0*

Table 10.6 Mean total number (±SEM) of Fos, ERα and double-labelled neurones (Fos + ERα) in two mPOA sections/ewe, three ARC sections/ewe and three VMN sections/ewe after saline and insulin treatment (n=6 ewes per group). $p<0.05$, between treatments within an area (*)

	ARC		VMN		mPOA	
	Saline	Insulin	Saline	Insulin	Saline	Insulin
Fos	165±13	313±24*	67±13	116±19*	185±35	146±29
ERα	626±104	790±172	862±86	817±156	1293±132	1229±117
Fos with ERα (%)	61±8	15±2*	82±3	69±8	39±11	26±7
ERα with Fos (%)	7±1	3±1*	7±1	22±2*	3±2	6±2

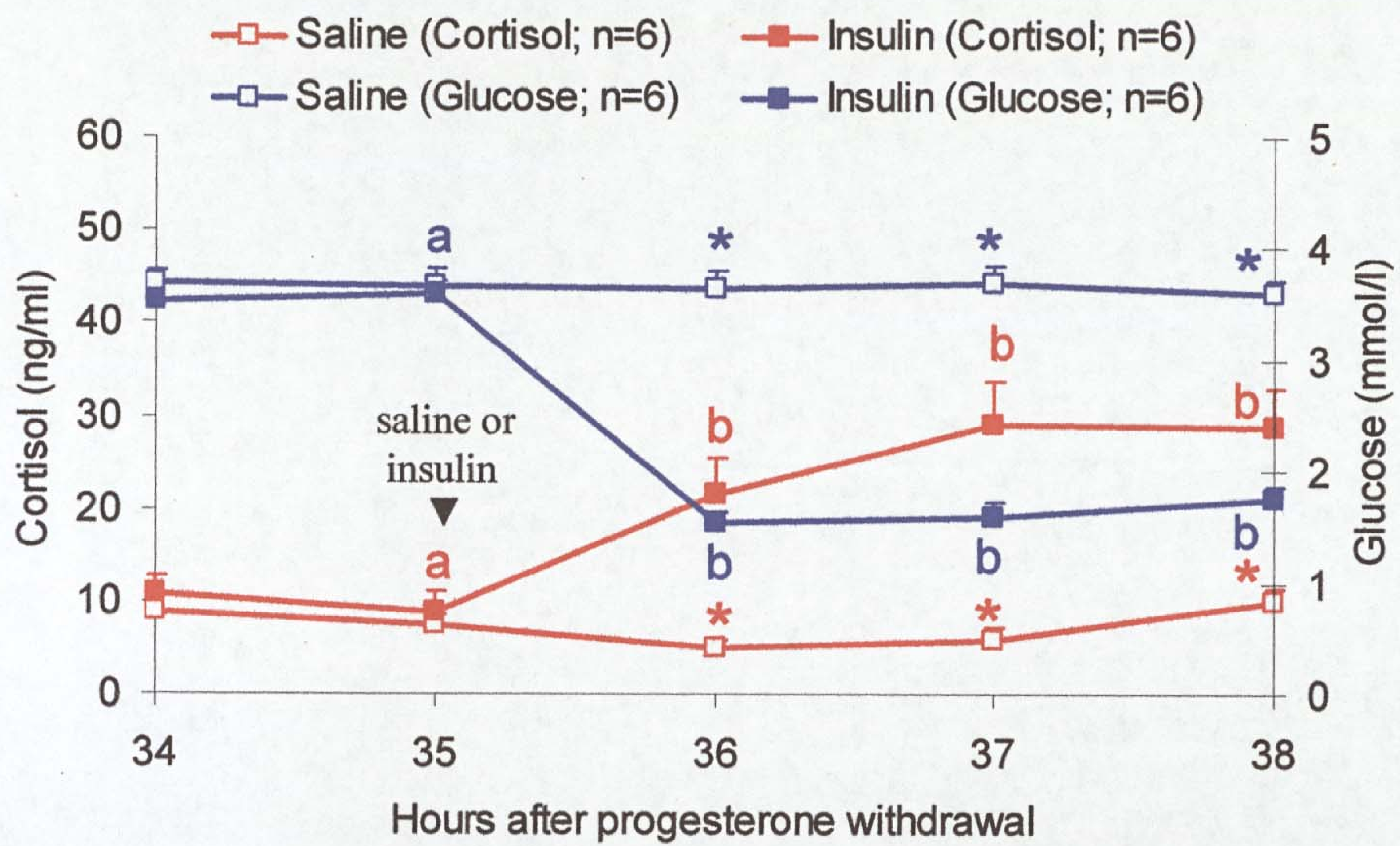


Fig 10.1 Mean (±SEM) plasma concentrations of cortisol and glucose from 1h before to 3h after intravenous injection of saline or insulin (5 IU/kg live-weight). $p<0.05$; within group (a *vs* b), between groups (*)

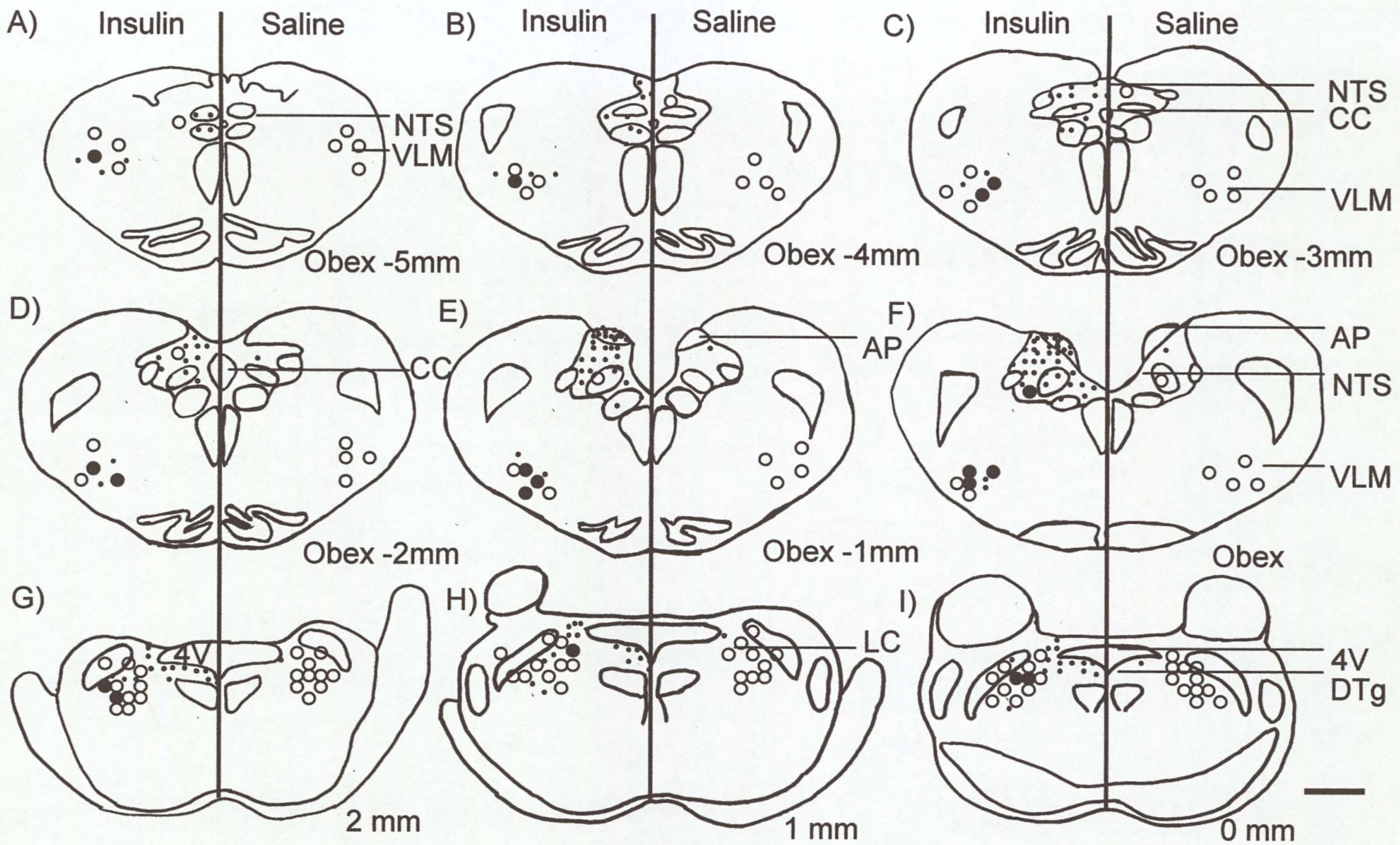


Fig 10.2 Schematic drawings of ewe brainstem coronal sections (A-I, caudal to rostral) examined for Fos (dots), DβH (empty circles) and double-labelled cells (filled circles). Each symbol represents approximately 5 cells. A-F) Caudal- and G-I) Rostral-brainstem, from caudal to rostral. Left or right half of each drawing indicates insulin or saline treated ewes, respectively. Abbreviations: AP, area postrema; cc, central canal; DTg, dorsal tegmental nucleus; LC, locus coeruleus; NTS, nucleus of solitary tract; VLM, ventrolateral medulla; 4V, fourth ventricle. Scale Bar, 5mm

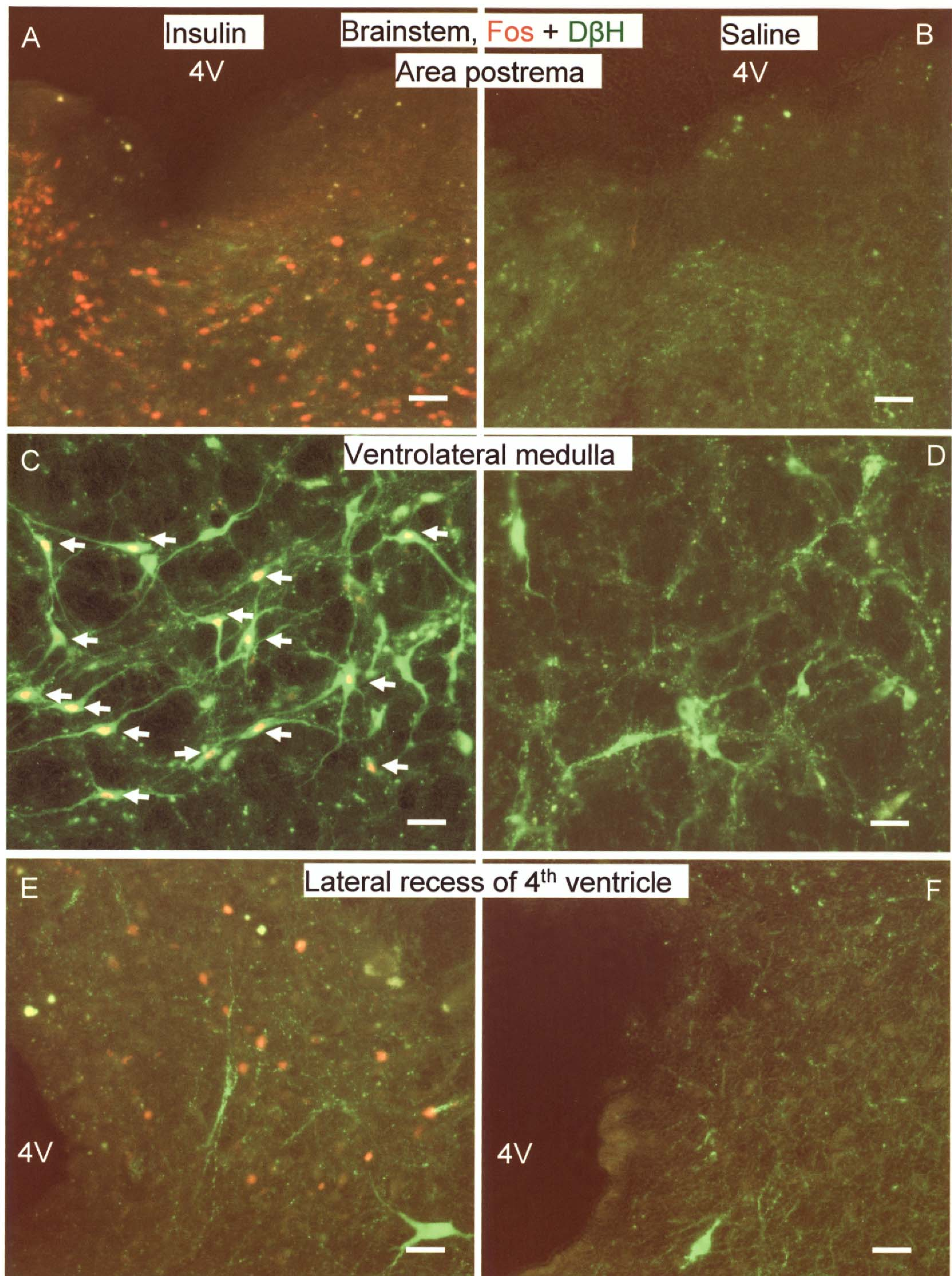
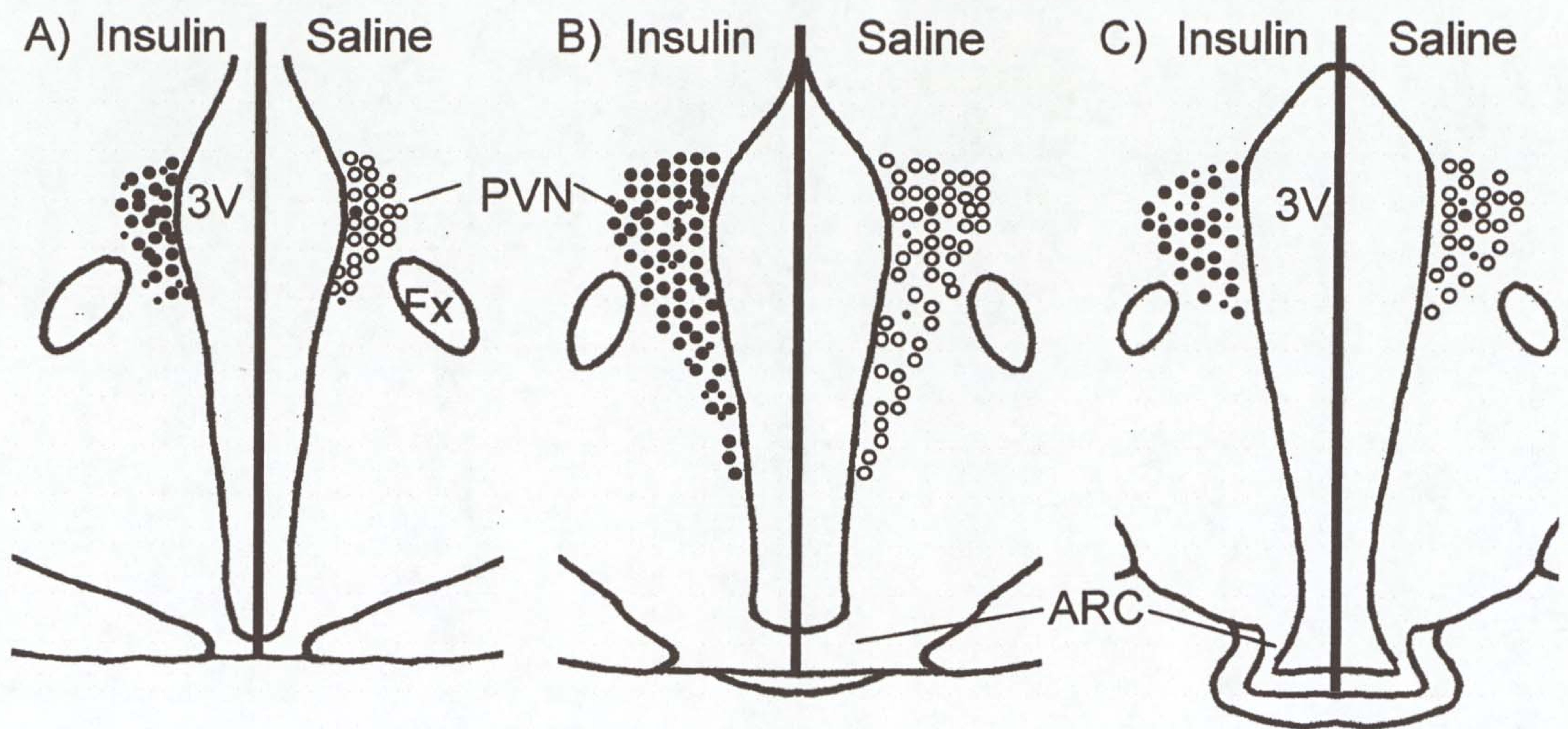


Fig 10.3 Representative images of caudal (area postrema and ventrolateral medulla) and rostral (lateral recess of 4th ventricle) brainstem showing Fos, noradrenergic (DβH), or Fos-positive noradrenergic (arrows) neurones in insulin or saline treated ewes. Scale bar, 50μm



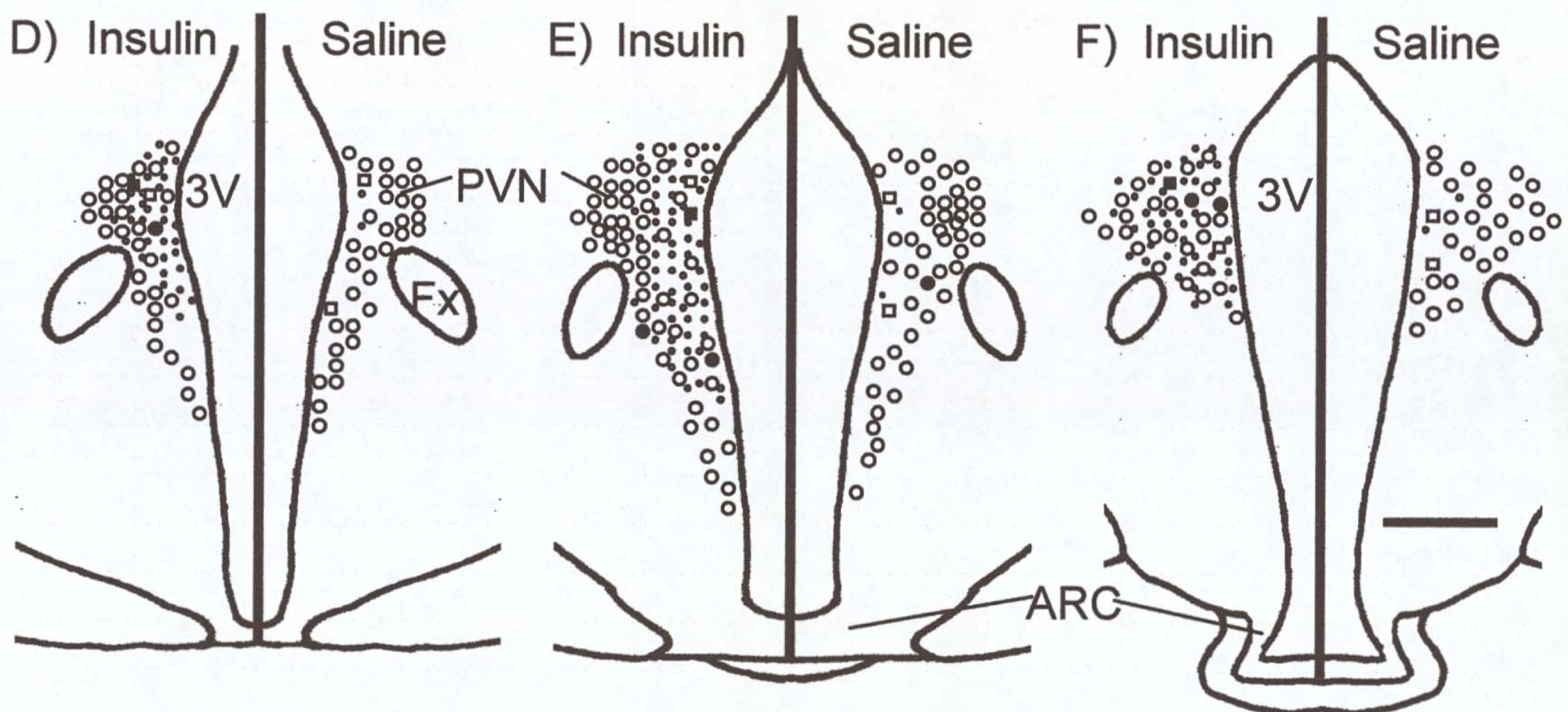


Fig 10.4 Schematic drawings of ewe hypothalamus coronal sections (560μm interval; rostral to caudal, A-C or D-F) examined for Fos (dots), CRH (A-C; empty circles), magnocellular AVP (D-F; empty circles) or parvocellular AVP (D-F; empty squares) and double-labelled cells (filled circles/squares). Each symbol represents approximately 5 cells. Left and right half of each drawing indicates insulin or saline treated ewes, respectively. Abbreviations: ARC, arcuate nucleus; Fx, fornix; PVN, paraventricular nucleus of hypothalamus; 3V, third ventricle. Scale Bar, 1mm

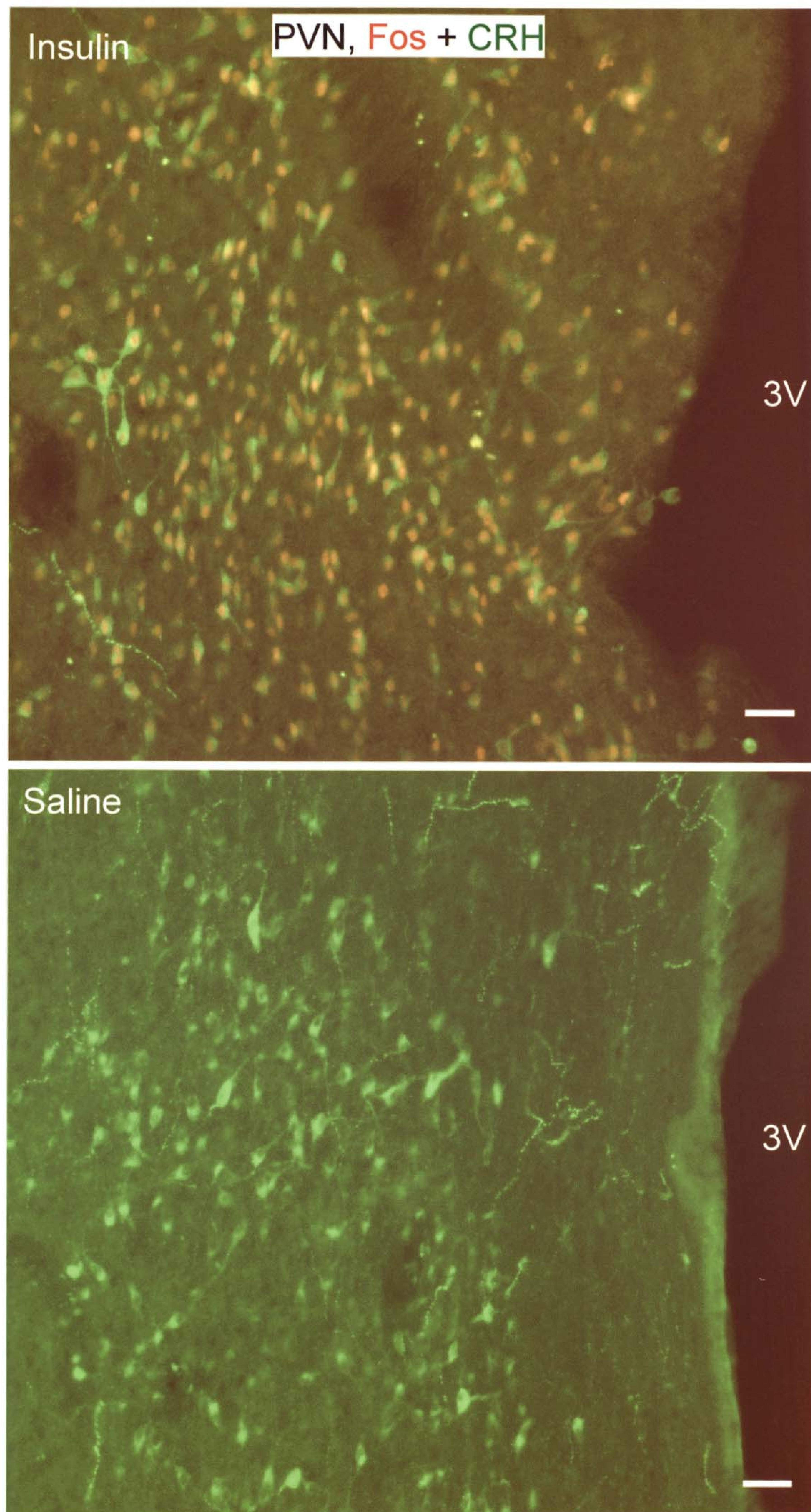


Fig 10.5 Representative images of the paraventricular nucleus (PVN) showing Fos, CRH, or Fos-positive CRH neurones in insulin or saline treated ewes. Almost all the CRH neurones are Fos-positive after insulin, but none in the saline treated ewes is Fos-positive in this section. Scale bar, 50μm

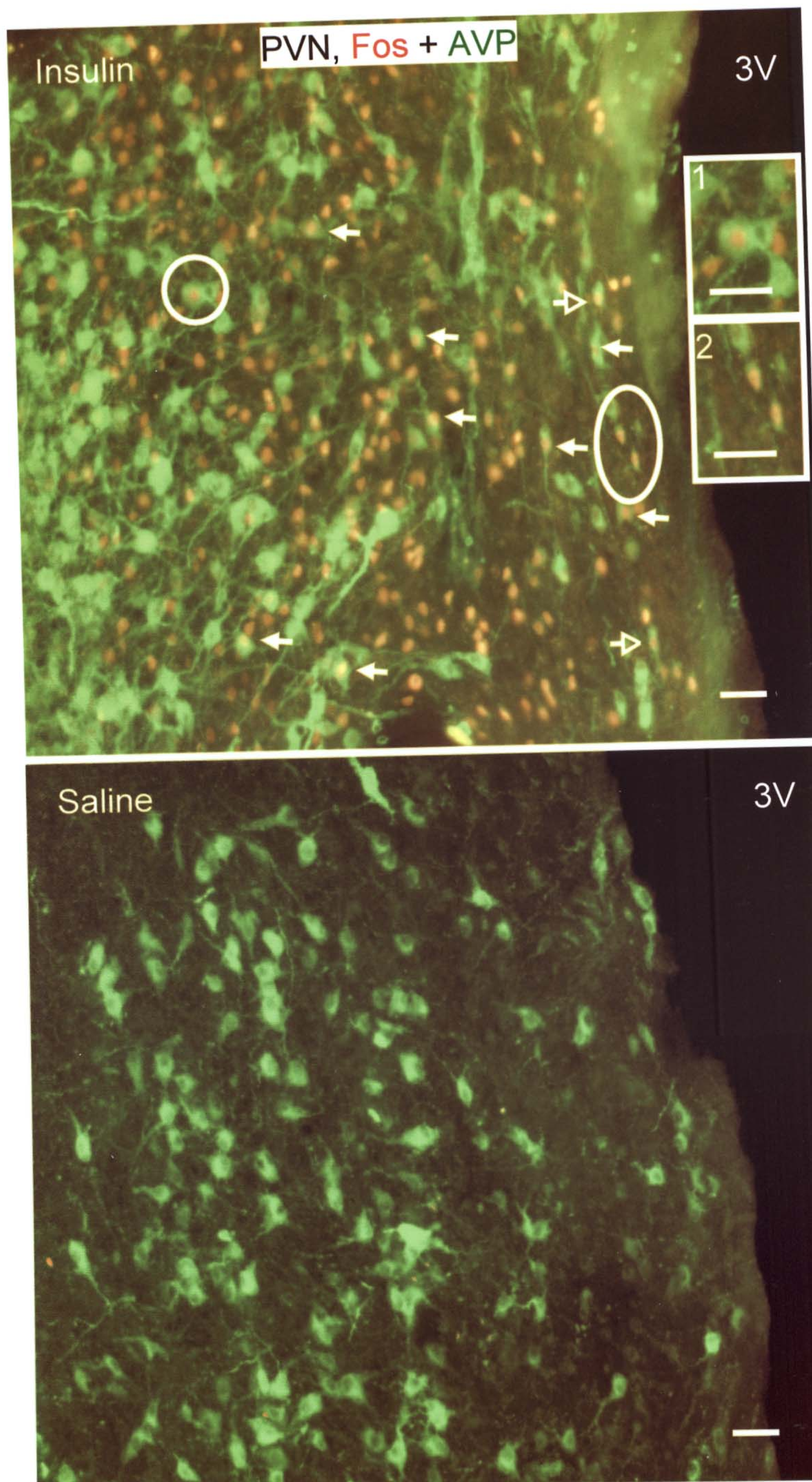


Fig 10.6 Representative images of the paraventricular nucleus (PVN) showing Fos, AVP, or Fos-positive AVP neurones in insulin or saline treated ewes. After insulin, both magnocellular (filled arrows and insert 1) and parvocellular (empty arrows and insert 2) AVP neurones are Fos-positive. None of the AVP neurones is Fos-positive in saline treated ewes in this section. Scale bar, 50µm

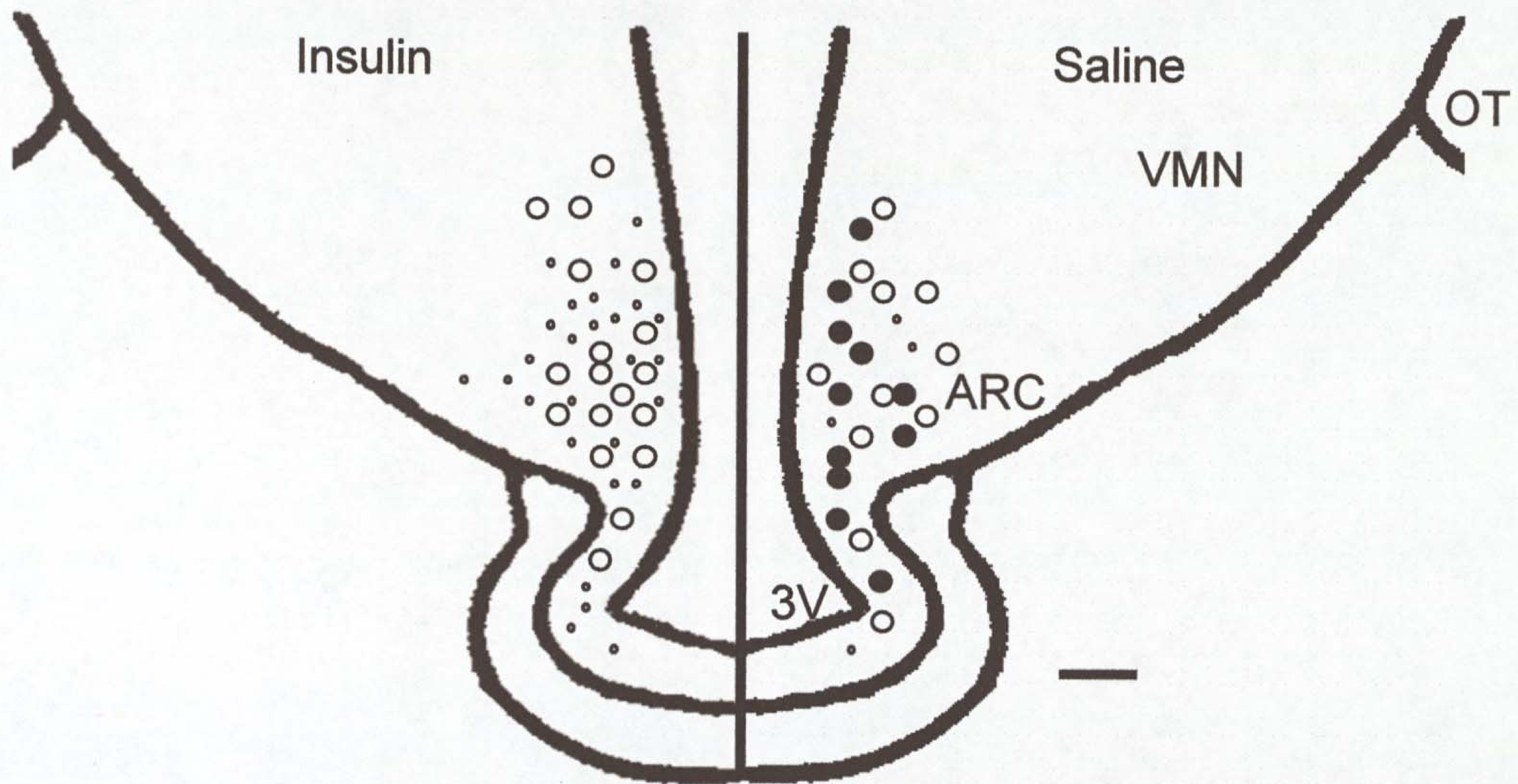


Fig 10.7 Schematic drawing of ewe hypothalamus coronal section examined for Fos (dots), β-endorphin (empty circles), and double-labelled cells (filled circles). Each symbol represents approximately 5 cells. Left and right half of each drawing indicates insulin or saline treated ewes, respectively. Abbreviations: ARC, arcuate nucleus; OT, optic tract; VMN, ventromedial nucleus; 3V, third ventricle. Scale Bar, 1mm

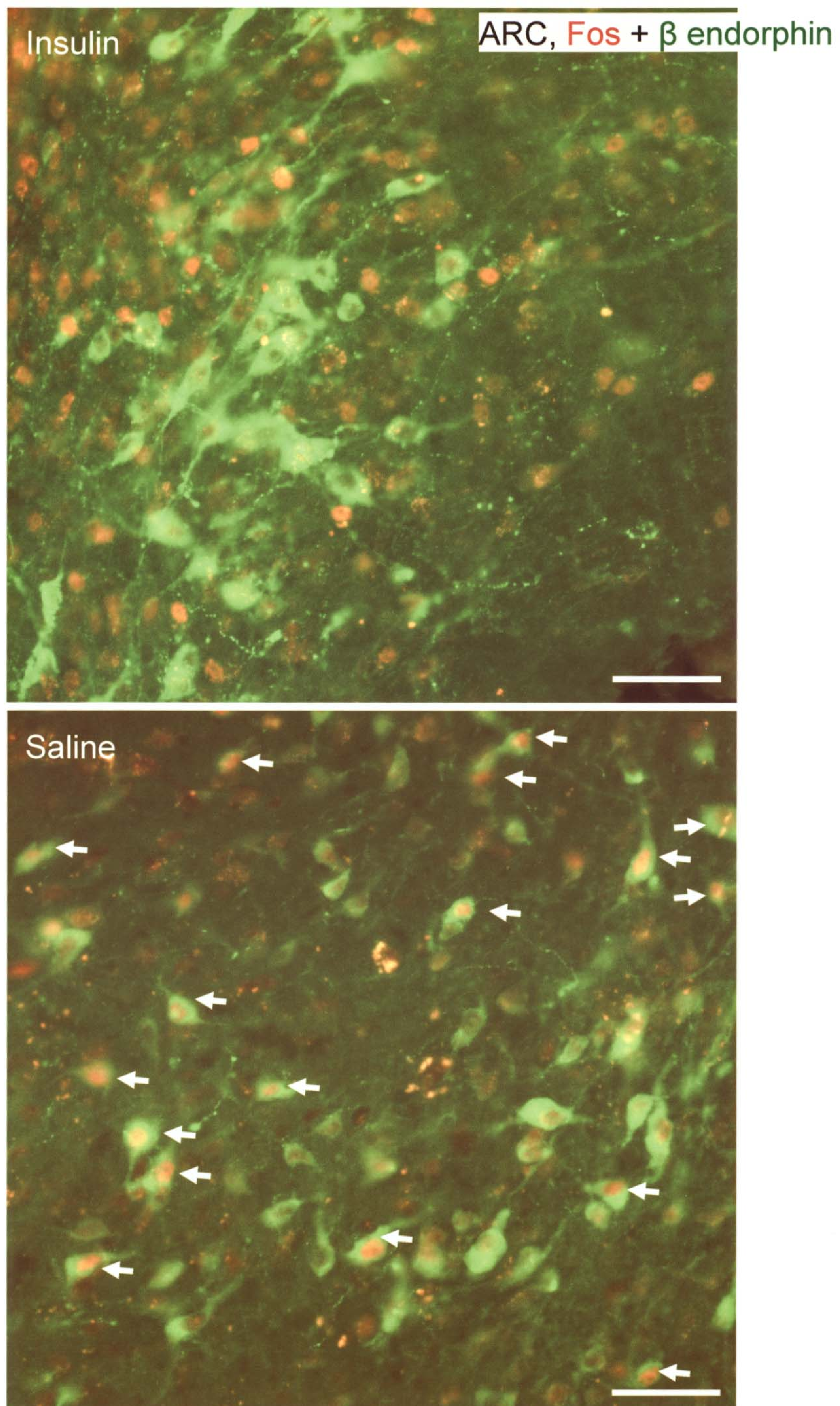


Fig 10.8 Representative images of the arcuate nucleus (ARC) showing Fos, β-endorphin, or Fos-positive β-endorphin (arrows) neurones in insulin or saline treated ewes. After insulin, Fos-positive neurones do not colocalise with β-endorphin though a number of Fos-positive β-endorphin neurones (arrows) are present in saline treated controls. Scale bar, 50μm

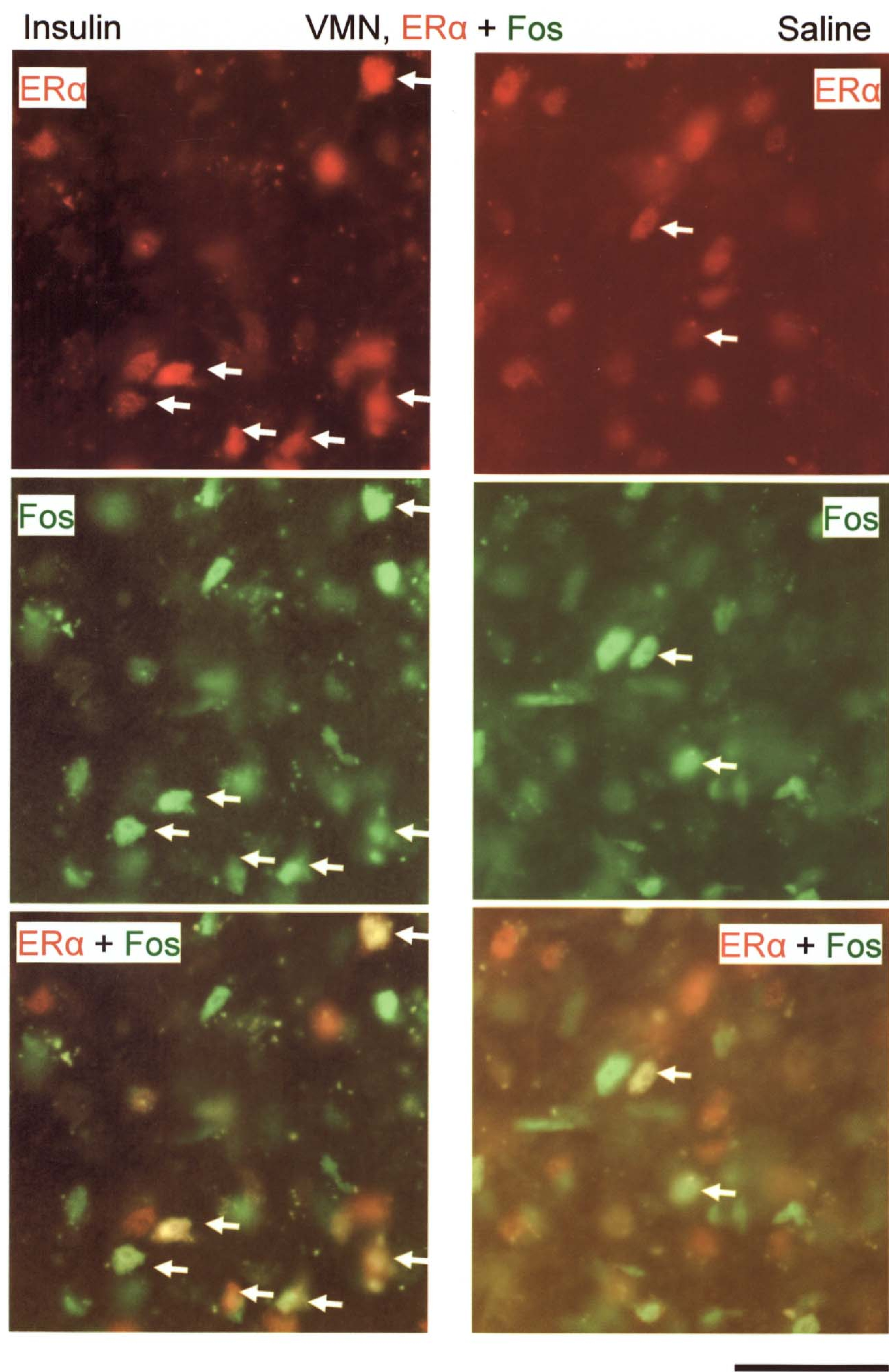


Fig 10.9 Representative images of the ventromedial nucleus (VMN) showing Fos, ERα, or Fos-positive ERα (arrows) cells in insulin or saline treated ewes. After insulin, Fos and ERα colocalisation increased in the VMN. Some of these lightly visible double-labelled cells are due to inability to bring all cells together in one focal plane. The proportion of the double-labelling (arrows) may not be in accordance with the text due to the small area shown. Scale bar, 50μm

mPOA, Fos + GnRH

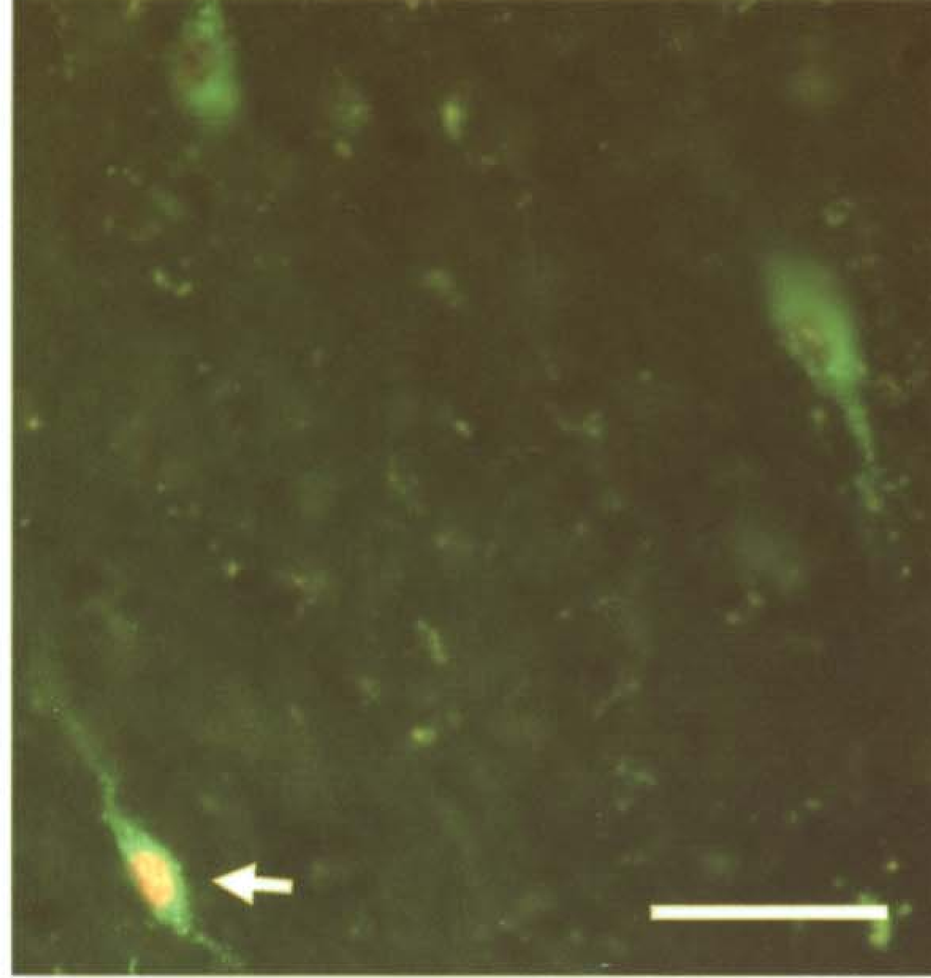


Fig 10.10 Representative image of the mPOA showing a Fos-positive GnRH neurone (arrow) in a saline treated ewe that had LH surge. Two Fos-negative GnRH neurones are also visible. Scale bar, 50μm

Chapter 11

General Discussion, conclusions and indications for future work

General Discussion, conclusions and indications for future work

General Discussion

The deleterious effect of stress on female reproductive function is now well established (Dobson *et al.*, 2003; Smith *et al.*, 2003b). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis and disrupts the hypothalamic-pituitary-gonadal (HPG) axis, specifically interrupting the release of gonadotrophin-releasing hormone (GnRH) and consequently luteinising hormone (LH). In addition to being the principal hypophysiotropic factors driving the HPA axis, corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) may be involved in the mechanism by which oestradiol potentiates suppression of the HPG axis. Hypothalamic γ-amino butyric acid (GABA) and brainstem noradrenergic neurones may also play key roles in the regulation of stress and reproduction axis interactions, especially in conjunction with oestradiol (E_2).

In this thesis *in vitro* and *in vivo* functional and neuroanatomical approaches were used to investigate interactions between stress and reproduction in the ewe brain.

In vitro perifusion system for hypothalamic slices (chapters 3 and 4)

In vitro hypothalamic perifusions were used to study interactions between neuroendocrine factors acting directly within the hypothalamus and thus avoiding confounding influences from the brainstem, pituitary, adrenal, ovarian, blood flow or metabolic processes. Due to technical problems with the CRH assay, only AVP and GnRH release in response to GABA or α_1-adrenoreceptor agonists or antagonists were investigated in different E_2 environments.

The functional viability of the hypothalamic slices was confirmed by increased release of both peptides in response to KCl. Furthermore, dampened GnRH responses to KCl in the presence of low E_2 but not high or no E_2 emphasized that the neuronal membranes were still sensitive.

Direct action of E_2 within the hypothalamus to augment AVP or GnRH release in vitro (chapter 3)

The greater release of basal AVP in the presence of high E_2 concentrations supports previous *in vivo* reports in sheep (Wood *et al.*, 2001) and *in vitro* studies in rats (Forsling, 1993). There are no ERα in AVP neurones in the ewe (chapter 9) and in the rat ERβ on AVP neurones inhibit AVP release (Shapiro *et al.*, 2000). So, there must be involvement of either interneurones or non-genomic mechanisms, as high E_2 induces exocytosis of AVP from rat magnocellular neurones *in vitro* (Wang *et al.*, 1995).

Basal release of GnRH was also stimulated by high E_2 concentrations in accordance with *in vivo* stimulation of GnRH release during the preovulatory period (Moenter *et al.*, 1990). GnRH neurones do not colocalise ERα in the ewe (Lehman & Karsch, 1993). Although there is evidence of ERα/β mRNA colocalisation with GnRH neurones in the mouse, it is still unclear whether E_2 has any direct influence on GnRH neurones (Skynner *et al.*, 1999). However, there is an indirect response to E_2 via the decreased stimulatory influence of noradrenaline on GABA function, and by increased adrenoreceptor coupling in the medial preoptic area (mPOA) to facilitate stimulation of GnRH neurones (Herbison *et al.*, 1990; Robinson *et al.*, 1991; Herbison, 1997b).

Our observations of increased AVP and GnRH release *in vitro* are in line with established *in vivo* findings. This emphasised the validity of our experimental approach and provided encouragement to further investigate interactions of GABA and noradrenergic systems with AVP and GnRH neurones within the hypothalamus of the ewe.

AVP neurones are under the inhibitory influence of GABA receptors with increased potentiation by E_2 through $GABA_B$ receptors in vitro (chapter 4)

Specific $GABA_{A \text{ or } B}$ antagonists revealed an inhibitory influence of GABA on AVP neurones within the hypothalamus, in agreement with others (Boudaba *et al.*, 1996; Slugg *et al.*, 2003; Li & Stern, 2004). Whether GABA receptors are present on AVP neurones is not known for ewe, however, AVP neurones do express GABA receptors in the rat (Fenelon & Herbison, 1995; Fenelon *et al.*, 1995; Slugg *et al.*,

2003). The present study provides evidence for GABA inhibition of AVP neurones via $GABA_A$ receptors in the absence of E_2, and via both GABA receptor subtypes in the presence of E_2.

AVP neurones are under the dual stimulatory-inhibitory control of α_1-adrenoreceptors with lower stimulatory effect in the presence of E_2 in vitro (chapter 5)

Noradrenergic neurones are well known to be stimulatory to AVP release via α_1-adrenoreceptors (Shioda *et al.*, 1997; Daftary *et al.*, 1998; Boudaba *et al.*, 2003), but recent reports also reveal α_1-adrenoreceptor-mediated inhibition of AVP neurones in the PVN (Han *et al.*, 2002; Iamova *et al.*, 2002; Chong *et al.*, 2004). In this area, 84% AVP neurones are in close contact with noradrenergic terminals (chapter 9), but the greater and prolonged response to α_1-adrenoreceptor antagonist (chapter 5) suggests net inhibitory control of AVP neurones.

The presence of α_1-adrenoreceptors on GABA neurones, which then synapse with AVP neurones in the rat PVN and SON (Theodosis *et al.*, 1986; Decavel *et al.*, 1989; Han *et al.*, 2002), along with the results obtained in chapter 4, enable a hypothesis to explain greater responses to the α_1-adrenoreceptor antagonist than the agonist in the presence of E_2. The agonist stimulation of α_1-adrenoreceptors on GABA neurones inhibits AVP neurones, so overcoming any stimulatory effects via α_1-adrenoreceptors directly on AVP neurones. After α_1-adrenoreceptor blockade (by the antagonist), the initial increase in AVP could have arisen by removal of strong inhibition by GABA. Later, the inhibitory GABA influence maintained by E_2, may override the α_1-adrenoreceptor antagonist-mediated increase in AVP, resulting in an early decline in AVP. In the absence of E_2, GABA influence was weak, through only $GABA_A$ receptors, and thus AVP remained elevated. This requires close contact between GABA terminals and AVP neurones in the PVN for which we found little evidence (chapter 9), but their existence can not be totally ruled out (discussed later, chapter 9).

Overall, E_2 appears to reduce the stimulatory effect of the α_1-adrenoreceptor agonist on AVP thus not supporting a role for AVP in E_2-induced potentiation of HPA axis activity via noradrenergic systems.

Cross reaction of GABA receptor (bicuculline) and α_1-adrenoreceptor (methoxamine) compounds in the GnRH assay (chapter 6)

Due to this unfortunate interaction, there was a set-back in continuing our *in vitro* experiments to investigate GABA and noradrenergic mechanisms controlling GnRH neurones within the hypothalamus (chapter 7 and 8). We tried to solve this problem by separating these interfering compounds from GnRH using a non-polar solid-phase extraction method. A wide range of columns with different concentrations of methanol (organic eluent) titrated to a pH range were evaluated. Regretfully, separation was not reproducible and accurate enough to be used routinely.

Oestradiol attenuates $GABA_A$ receptor-mediated inhibitory control of GnRH neurones within the hypothalamus in vitro (chapter 7)

The net increase in immunoreactive GnRH release suggests predominant $GABA_A$ receptor-mediated inhibitory control on GnRH neurones. In the POA, there are close contacts between GnRH cell bodies and GABA terminals, and GnRH neurones colocalise $GABA_A$ receptors (Jung *et al.*, 1998; Jansen *et al.*, 2003).

In the presence of high E_2, no difference was observed in response to $GABA_B$ compounds. The hypothalamic slices were collected in January and previous work suggests that during the breeding season $GABA_A$ but not $GABA_B$ receptors mediate GnRH release by E_2, and secondly $GABA_B$ receptors are not involved in GnRH release mechanisms (Scott & Clarke, 1993a; Scott & Clarke, 1993b; Jackson & Kuehl, 2004).

Oestradiol potentiates α_1-adrenoreceptor stimulation of GnRH neurones within the hypothalamus in vitro (chapter 8)

Oestradiol potentiated the stimulatory effect of an α_1-adrenoreceptor agonist on net GnRH release, supporting established theories (Demling *et al.*, 1985; Domanski *et al.*, 1991). The existence of Fos-immunoreactivity in brainstem noradrenergic neurones that colocalise ERα and the release of noradrenaline in the POA during an E_2-induced GnRH surge both highlight the involvement of the brainstem during

stimulation of the noradrenergic system by E_2 (Clarke *et al.*, 1999; Scott *et al.*, 1999; Rawson *et al.*, 2001). However, the present study extends these observations to show that E_2 can stimulate GnRH neurones without brainstem involvement.

Oestradiol modifies noradrenergic-GABA interactions in the mPOA. In this area, there is noradrenergic input to GABA neurones, that in turn colocalise α_1-adrenoreceptors and project terminals to GnRH neurones (Leranth *et al.*, 1988a; Han *et al.*, 2002; Pompolo *et al.*, 2003a). Studies in the rat and ewe suggest that when E_2 is low noradrenaline stimulates GABA, but in the presence of high E_2 as in the preovulatory period, noradrenaline fails to activate GABA neurones (Herbison *et al.*, 1990; Robinson *et al.*, 1991; Herbison, 1997a).

All these observations, along with our own, suggest that in the presence of E_2 noradrenaline can act on GnRH neurones via increased α_1-adrenoreceptor function. As well as via the indirect route by which E_2, acting through α_1-adrenoreceptors, induces a decrease in $GABA_A$ function, and thus reduced activation of GABA neurones. This ultimately lifts tonic GABA inhibition of GnRH neurones.

Relationship of noradrenergic and GABA terminals with CRH and AVP cell bodies in the PVN and with β-endorphin cell bodies in the ARC (chapter 9)

Close contacts were observed between noradrenergic terminals and CRH or AVP cell bodies in the PVN, and others have shown that these projections originate from noradrenergic neurones in the brainstem (Cummings & Seybold, 1988; Cunningham & Sawchenko, 1988; Ginsberg *et al.*, 1994). These contacts are functionally important in ewe as noradrenaline stimulates both CRH and AVP release *in vivo* (Liu *et al.*, 1991), and modulates AVP release via α_1-adrenoreceptors *in vitro* (chapter 5).

Close contacts were observed between GABA neurones and CRH, but not AVP, cell bodies in the PVN. The inhibition of AVP release by GABA receptor compounds *in vitro* (chapter 4) questions the absence of these close contacts. However, in rat, both CRH and AVP cell bodies receive GABA innervation in the PVN and GABA receptor subtypes are present on AVP neurones in the PVN (Decavel *et al.*, 1989; Fenelon & Herbison, 1995; Fenelon *et al.*, 1995; Miklos & Kovacs, 2002;

Slugg *et al.*, 2003). In the present study, GABA terminals were in very close proximity to AVP cell bodies and might exert influence via diffusion.

In the ARC, there was no close interaction between noradrenergic terminals and β-endorphin cell bodies, however, GABA terminals were in close contact, indicating that β-endorphin may not be under stimulatory noradrenergic influence but could be restrained by inhibitory GABA neurones.

Relationship of CRH, AVP and β-endorphin terminals with GnRH cell bodies in the mPOA and with GnRH terminals in the ME (chapter 9)

CRH, AVP and β-endorphin terminals were not very close to GnRH cell bodies in the mPOA, with exception of only one close contact of β-endorphin. However, abundant CRH and β-endorphin terminals in the mPOA suggest a role in this area but other interneurone(s).may be involved.

In the external zone of the median eminence, there was close association between GnRH and CRH terminals suggesting this as one of the sites for stress-induced suppression of GnRH. In support, *in vivo* administration of CRH to the ME suppresses LH in other species (Ortega *et al.*, 1994; Frias *et al.*, 1997).

No interaction was evident between AVP or β-endorphin and GnRH terminals in the ME even though infusion of β-endorphin into the ME can suppress GnRH release in the ewe (Conover *et al.*, 1993).

Oestradiol through ERα may directly act on brainstem noradrenergic and ARC β-endorphin neurones but not on CRH and AVP neurones in the PVN (chapter 9)

The presence of ERα in brainstem noradrenergic neurones agrees with previous studies (Simonian *et al.*, 1998; Scott *et al.*, 1999). There are noradrenergic projections from this area to the PVN in the rat (Cunningham & Sawchenko, 1988). This evidence, and presence of close contacts between noradrenergic terminals and CRH or AVP cell bodies in the PVN, suggests that E_2 may potentiate stress-induced suppression of HPG axis through brainstem neurones. However, ERα colocalising noradrenergic neurones also project from the same brainstem nuclei to the mPOA and are thought to be involved in GnRH surge generation in the ewe (Clarke *et al.*, 1999;

Scott *et al.*, 1999; Rawson *et al.*, 2001). It is not known whether during stress, noradrenergic neurones related to stress override the mechanisms of surge-stimulatory noradrenergic neurones. Nevertheless, immunodestruction studies in rat suggest that separate noradrenergic populations in the brainstem are involved in the normal oestrous cyclicity or the inhibition of reproductive functions (I'Anson *et al.*, 2003).

In the PVN, the absence of ERα in CRH and AVP neurones suggests that these neurones are not influenced directly via ERα to potentiate the HPA axis. However, there could be non-genomic action as E_2 induces an increase in AVP release *in vitro* (chapter 3).

However, E_2 may modulate the stress axis via β-endorphin neurones that colocalise ERα in the ARC. An increase in the activity of β-endorphin neurones in a high E_2 environment is well known (chapter 10)(Domanski *et al.*, 1991; Conover *et al.*, 1993).

Insulin treatment increases Fos-immunoreactivity in noradrenergic neurones in the caudal brainstem, and CRH and AVP neurones in the PVN, but decreases Fos in β-endorphin neurones in the ARC (chapter 10)

The increase in Fos-positive cells in the AP is due to low glucose (Adachi *et al.*, 1995) and this region is also involved in disruption of GnRH release during low glucose availability in the rat (Cates & O'Byrne, 2000).

Considerable numbers of Fos-positive noradrenergic neurones were observed in the NTS and VLM after insulin. This response is probably related to initiation of HPA activity via the PVN neurones with eventual suppression of the HPG axis (Nagatani *et al.*, 1996; I'Anson *et al.*, 2003). In the NTS, Fos was also observed in non-noradrenergic neurones after insulin treatment, the phenotype of some of these neurones projecting to the PVN is unknown but could be related to activation of the HPA axis (Iyengar *et al.*, 1987; Kawano & Masuko, 1996; Kinzig *et al.*, 2003).

The presence of Fos in almost all the CRH neurones in the PVN after insulin could be one pathway through which insulin suppresses GnRH, as terminals of both CRH and GnRH neurones converge in the same region of the ME (chapter 9). However, CRH administration to unstressed sheep failed to suppress LH, although in

stressed sheep CRH increased coincident with GnRH suppression (Clarke *et al.*, 1990; Caraty *et al.*, 1997; Battaglia *et al.*, 1998). In the rat, CRH is involved in LH suppression after low glucose but intriguingly destruction of the PVN does not obliterate effects on LH suppression after foot-shock or interleukin treatment (Rivest & Rivier, 1991; Maeda *et al.*, 1994). One possibility is that different central pathways are involved in activation of HPA activity depending upon the stress-stimulus. For example, elimination of brainstem noradrenergic neurones projecting to PVN impairs CRH expression and HPA activity after glucoprivation, but had no effect on PVN responses to swim stress (Ritter *et al.*, 2003).

The other possibility is that areas other than the PVN could also be involved in stress-induced suppression of GnRH. We have observed alterations in the ERα and Fos-colocalising populations in the ARC and VMN after insulin (chapter 10), disruption of which may affect the normal E_2-induced surge-generating signal for GnRH neurones. Secondly, inhibitory neurotransmitters may be activated in these areas which could influence GnRH neurones without PVN involvement. Also, it is known in the rat that stress-specific brainstem noradrenergic neurones project to PVN (Cunningham & Sawchenko, 1988), but it remains unclear whether stress-related noradrenergic neurones project to the mPOA to directly inhibit GnRH neurones or to activate inhibitory neurotransmitters which could further suppress GnRH (Rivier & Rivest, 1991). Evidence is also available for noradrenergic innervation of the inhibitory GABA neurones in the PVN (Leranth *et al.*, 1988a).

In addition to the PVN, CRH terminals could originate from the mPOA and the amygdala where substantial number of CRH cell bodies are present in the rat (Hahn *et al.*, 2003; Herman *et al.*, 2003).The identity and the involvement of these non-PVN CRH terminals remain unclear in the ewe. Recently, a role for CGRP neurones (abundant in the rat mPOA) has been suggested during insulin-induced suppression of the GnRH release (Li *et al.*, 2004).

Overall, all this evidence suggests that although there is substantial activation of PVN neurones after insulin treatment, effort is required to determine whether the PVN is the only source of HPA activation and ultimately GnRH suppression, or does

the latter involve multiple feedback mechanisms through various hypothalamic and brainstem nuclei?

A subpopulation of both magnocellular and parvocellular neurones were Fos-positive after insulin but neither GnRH cell bodies nor terminals were associated with AVP terminals (chapter 9). Indeed in one study, administration of AVP did not suppress LH (Clarke *et al.*, 1990).

After insulin, the marked decrease in the number of Fos-positive β-endorphin neurones may be related to disruption of inhibitory tone on GnRH neurones, or to removal of opioid inhibitory tone on PVN neurones potentiating HPA axis activity. The precise role of these insulin-suppressed β-endorphin neurones remains to be determined. In the rat, β-endorphin neurones project from the ARC to the PVN and β-endorphins do suppress CRH release (Yajima *et al.*, 1986; Tsagarakis *et al.*, 1990; Baker & Herkenham, 1995). GABA terminals, of unknown origin, were in close contact to β-endorphin neurones (chapter 9) and following insulin GABA turnover increased in the nearby ventromedial nucleus (VMN) (Beverly *et al.*, 2001). Whether GABA neurones of the VMN send projections to ARC β-endorphin neurones needs to be determined.

Insulin treatment decreases Fos-immunoreactivity in ERα-positive cells in the ARC, in contrast to an increase in the VMN, and no alteration in the mPOA (chapter 10)

In the ARC, subpopulations of inhibitory β-endorphin and NPY neurones, excitatory glutamate neurones and neurokinin-B neurones of unknown role all colocalise with ERα (Lehman & Karsch, 1993; Skinner & Herbison, 1997; Caraty *et al.*, 1998b ; Goubillon *et al.*, 2000; Grindrod *et al.*, 2002; Estrada *et al.*, 2003; Pompolo *et al.*, 2003b). A decrease in β-endorphin neurone activity in the present study (chapter 10) was associated with an increase in unidentified Fos-positive cells. The nature and role of these cells needs clarification.

In the VMN after insulin treatment, there was an increase in the number of both ERα-positive and non-ERα positive cells colocalising Fos, the identity of which is not clear although GABA may be involved as turnover of this neurotransmitter is increased in the VMN both by E_2 and by insulin (Luine *et al.*, 1997; Beverly *et al.*,

2001). Considering the importance of the VMN in relation to generation of the GnRH surge (Caraty *et al.*, 1998a), further investigation of how insulin affects ERα colocalising populations in this area is warranted.

GnRH neurones are Fos-positive only during the surge-release phase (chapter 10)

The present study shows that GnRH neurones are not Fos-positive during the follicular transmission phase. Frequent measurements of GnRH in portal blood reveal very low concentrations with increasing pulse frequencies 2-5 hours before GnRH surge initiation. For approximately two hours before surge onset basal GnRH concentrations also increase (Moenter *et al.*, 1991). However, in the present study it is not possible to know whether a GnRH surge was imminent when the ewes were sacrificed. Similarly, the number of Fos-positive GnRH neurones markedly increased only during the surge-release phase, in addition to E_2-induced Fos in non-GnRH cells (Moenter *et al.*, 1993). We confirmed that some of these Fos-colocalising non-GnRH cells were ERα-positive, suggesting that they are part of E_2-sensitive neuronal chain that leads to GnRH surge generation.

The reason for the absence of Fos in GnRH neurones before the onset of GnRH surge could be explained as follows. Firstly, the detection of Fos could require a magnitude of stimulation that was not achieved by GnRH neurones before the onset of the GnRH surge. Secondly, the presence of Fos in GnRH neurones only at the time of the surge could suggest that increased afferent neuronal stimulation of GnRH neurones initiates the GnRH surge, and not synchronisation of ongoing disordered activity. If more and more GnRH neurones were recruited into an active state before surge onset then some GnRH neurones should be firing and expressing Fos before the onset of the GnRH surge. Overall, this would suggest that surge onset occurs at the end of the interneuronal transmission of the E_2 signal to GnRH neurones, and not the ongoing recruitment of GnRH neurones to finally culminate in surge release.

Conclusions

*The E_2 stimulation of basal AVP directly from the *in vitro* hypothalamus favours E_2-potentiation of HPA activity. However, this contrasts with E_2-mediated suppression of AVP release revealed by responses to GABA and α_1-adrenoreceptor compounds. It is possible that in this *in vitro* system, E_2 predominantly acts via non-neuronal mechanisms to slightly elevate basal AVP release. However, neuronal mechanisms are also operational as revealed by exposure to GABA and α_1-adrenoreceptor compounds. Differential suppression of AVP release in the presence of E_2 occurs by interactions between the GABA and noradrenergic systems

*Differential responses of the GnRH system to GABA antagonist and α_1-adrenoreceptor agonist in the presence or absence of E_2 suggest that E_2 potentiates GnRH release from the hypothalamus *in vitro*. These results are in line with the *in vivo* mechanism of the surge-induction process during preovulatory period when E_2 concentrations are high.

*A part of the neuroanatomical framework of the stress axis involves noradrenergic and/or GABA input to CRH, AVP and/or β-endorphin neurones, the output of which could influence the reproduction axis through interactions between GnRH and CRH terminals in the ME. However, there are other sites of interaction of the stress axis at the level of GnRH cell bodies. For example, in the mPOA, there are CRH, β-endorphin and AVP terminals which could interact with GnRH cells even through involvement of one or two interneurones. The importance of these interactions between stress-related terminals and GnRH cell bodies in the mPOA lies in the inhibition of GnRH pulse frequency.

*Insulin-induced disruption of the GnRH surge involves a complex system of neurones in the NTS and VLM of the caudal brainstem and the paraventricular, arcuate and ventromedial nuclei of the hypothalamus. After insulin, Fos-positive brainstem noradrenergic neurones could stimulate PVN neurones to activate HPA axis. The inhibition of GnRH neurones following stress could also result from the disruption of Fos-positive ERα-colocalising populations that form part of the neural chain sending the E_2-signal to GnRH neurones.

Indications for future work

*There is need for the modification of the *in vitro* perifusion system in terms of developing the microinjection technique. For this even better slice collection procedures need to be adopted to maintain intact neuronal integrity throughout the slice thickness.

**In vitro* microinjection of various neurotransmitter compounds into the ME, ARC, VMN, PVN or mPOA might delineate the specific hypothalamic areas and the neurotransmitters involved in the regulation of GnRH or AVP release.

*The interactions between noradrenergic and GABA systems in differing oestradiol environments need to be explored further in the *in vitro* system.

*There is need to understand the interactions between GnRH and other neurotransmitters in the presence of progesterone and/or glucocorticoids in the perifusion media, to simulate the *in vivo* milieu of these steroids in normal and stress conditions.

*Using OVX and E_2-treated OVX animals, there is need to explore the interactions of the different neurotransmitters with the CRH and/or AVP neurones to delineate E_2-sensitivity of the HPA axis.

**In vivo*, there is an urge to delineate the origin of the GABA and noradrenergic terminals in the PVN by tract tracing studies.

*Immunodestruction of brainstem noradrenergic neurones by retrograde transport from the PVN or the mPOA in normal and stressed animals could delineate the role of these neurones in GnRH surge disruption.

*Colocalisation studies of CRH and AVP neurones in the PVN would give further insight into the relative importance of these neurones in activation of HPA axis.

*Any interaction of ARC β-endorphin neurones with the CRH and/or AVP neurones need to be determined by confocal studies and how β-endorphins controls CRH and/or AVP release could be determined by *in vitro* system.

*Another important question remaining to be answered is the *in vivo* role of β-endorphin neurones on PVN activity. Whether β-endorphin neurones have tonic inhibitory control over the PVN activity could be determined by Fos studies in normal and stressed animals at times other than the follicular phase.

*How β-endorphin neurones are restrained following stress needs to be investigated. Microinjection of specific GABA receptor compounds in the ARC and/or VMN could give a clue. Also, in addition to insulin, the activity of β-endorphin neurones after other types of stressors needs to be determined.

*Investigating the role of the other opioids, dynorphin and/or enkephalin during follicular phase and following stress could decide whether all opioids function in same way or are different from β-endorphin.

*As GABA neurones provide input to GnRH cell bodies, when antisera staining for GABA cell bodies become available we could determine whether CRH, AVP or β-endorphin terminals in the mPOA act on the GnRH neurones through GABA interneurones.

*With triple-labelling, the identity of the ERα populations colocalising Fos during stress could delineate the exact GnRH surge-generating mechanisms in the ARC and VMN area interrupted by stress.

*With neurotoxins, destruction of the CRH and/or AVP neurones in the PVN could answer if these are involved in surge disruption mechanisms or not in the stressed animals. An alternative possibility is the central use of CRH and/or AVP antagonists to understand these mechanisms.

*In an OVX ewe model, sacrificing the ewes at regular intervals after the surge-inducing E_2 signal to identify Fos-colocalising neurones in various hypothalamic nuclei may point to various neurones involved in the stages of activation, transmission or surge-release.

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Oestradiol (E_2) stimulates the release of GnRH and arginine vasopressin from the sheep hypothalamus *in vitro*

SPS Ghuman[1], S Prabhakar[1], RF Smith[1] & H Dobson[1]

[1]Department of Veterinary Clinical Science, University of Liverpool, Leahurst, UK

spghuman@liv.ac.uk

INTRODUCTION

Oestradiol (E_2) sensitises stress responses of the reproductive axis *in vivo*

Our current aim is to investigate whether E_2 influences GnRH and arginine vasopressin (AVP) release at the hypothalamic level

This was achieved by developing a novel *in vitro* perifusion system in which hypothalamic slices were continuously exposed to media containing low or high E_2 (Oestradiol 17-β)

PROCEDURES

Slice collection procedures:

At a local abattoir, brains were removed from the cranial cavity within 10min of death.

Using skin graft blade, saggital midline hypothalamic slices (from anterior preoptic area to rostral edges of mammillary bodies, 2mm thick; 2 per sheep) were dissected and placed in glass vials containing ice-cold oxygenated perifusion media (MEM-∝; Sigma) alone (control); with low (6pg/ml) or high E_2 (24pg/ml)

Perifusion procedures:

Within 2h of death, hypothalamic slices (per ewe distributed between groups) were transferred to 12 separate perifusion chambers (Polycarbonate syringe filter holders; Internal vol-1.5ml) perifused at 37°C with oxygenated MEM-∝ (pH7.4; Flow rate 0.15 ml/min) alone, with low or high E_2

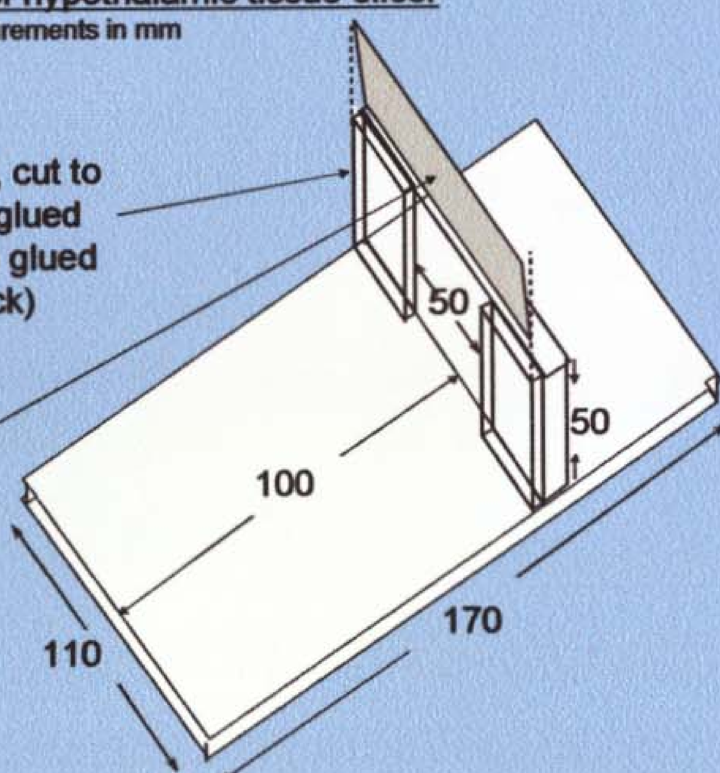
After 5h stabilisation, 10min fractions were collected for 3h. Functional viability of slices was confirmed by exposure to depolarising dose of KCl (100mM) for 10min in the last hour of perifusion

Analyses:

Concentrations of GnRH and AVP were measured by radio immunoassay. The obtained data were processed statistically using GLM ANOVA. Mean GnRH and AVP during the 2h period immediately before KCl challenge were considered as basal

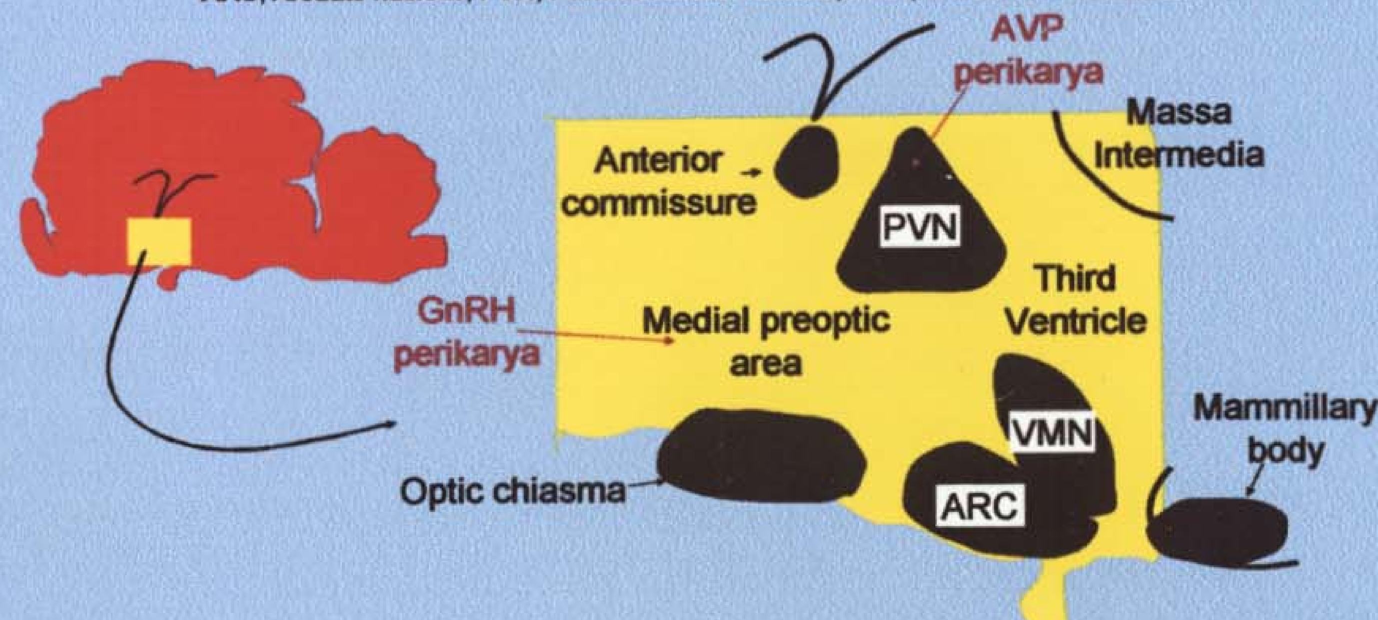
Schematic diagram of hypothalamic tissue slicer

measurements in mm

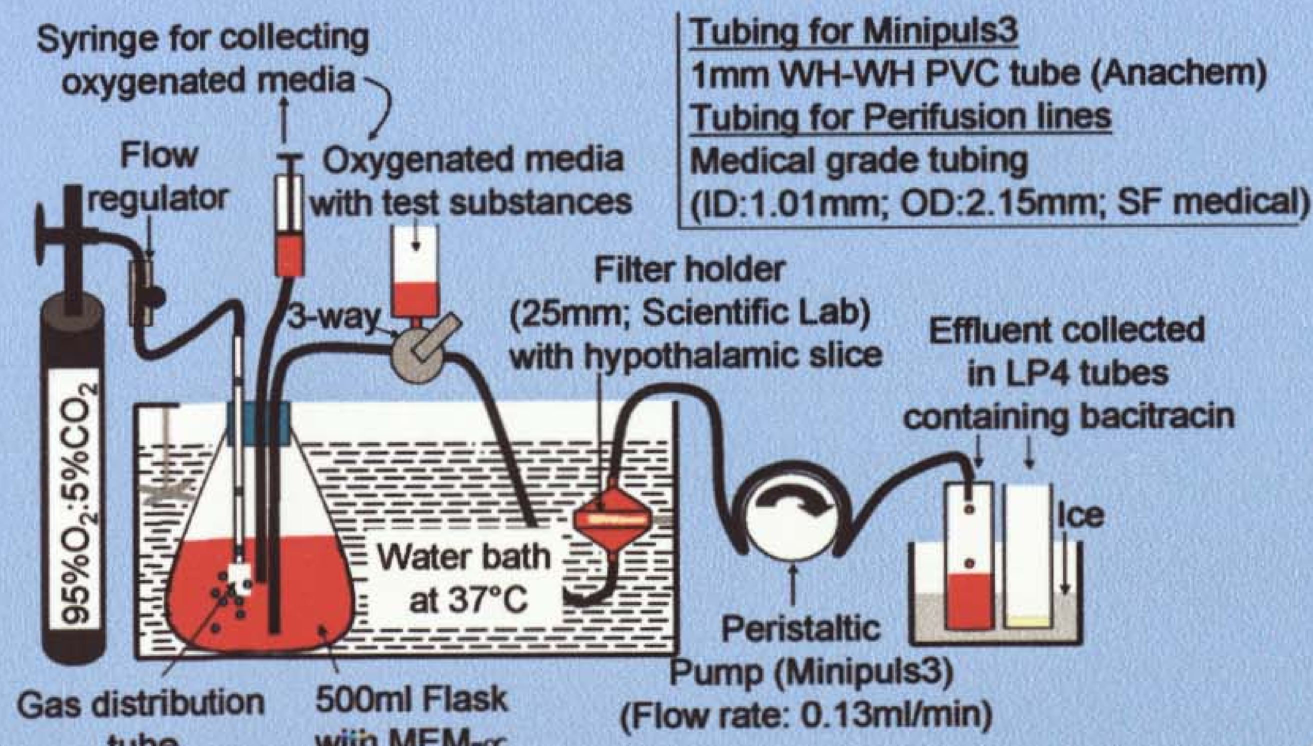


A saggital section of sheep brain showing the areas of hypothalamic slice

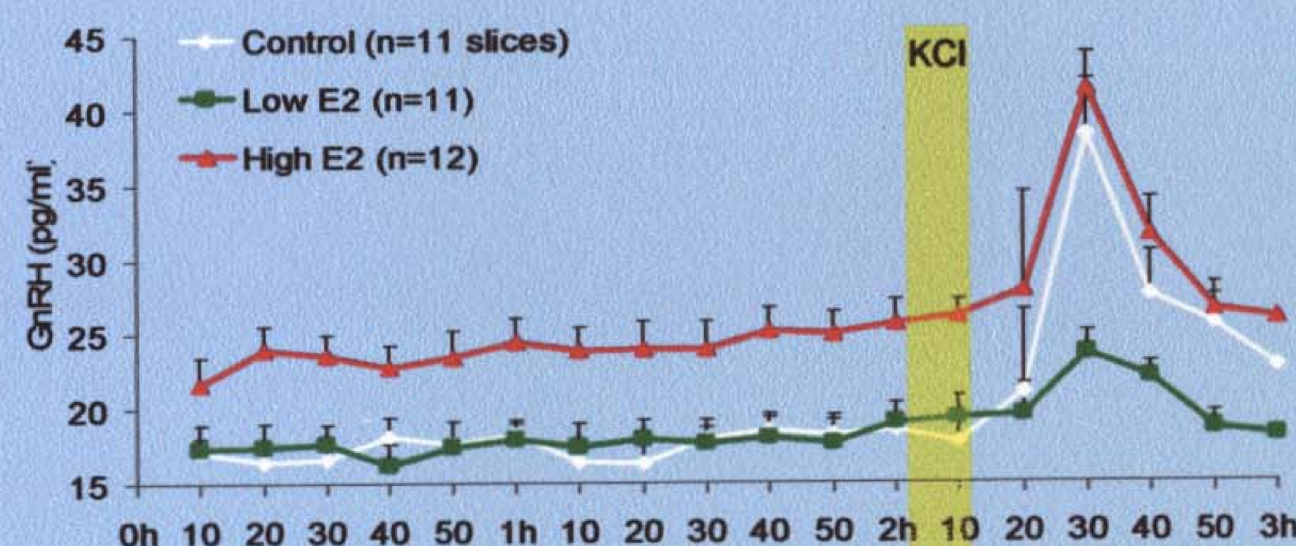
ARC, Arcuate nucleus; PVN, Paraventricular nucleus; VMN, Ventromedial nucleus

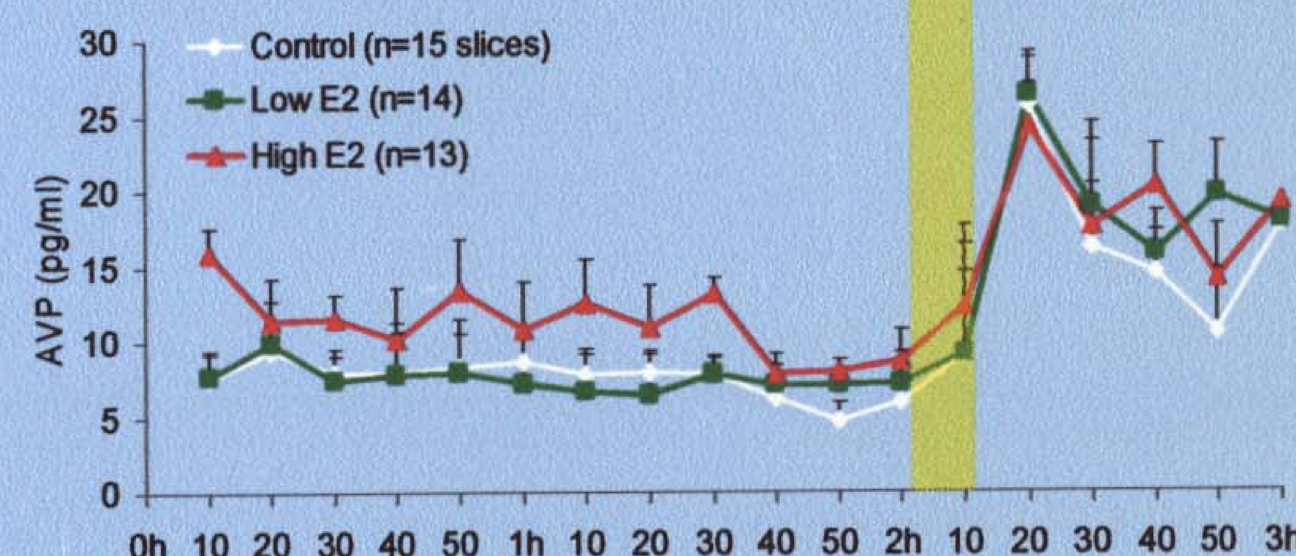


Diagrammatic representation of the perifusion assembly



RESULTS





Responses of hypothalamic slices to low E_2 were equivocal for GnRH (p=0.9) and AVP (p=0.8). However, high E_2 increased basal GnRH (p=0.001) and AVP (p=0.001)

After KCl, GnRH and AVP increased significantly (p=0.001) compared with the value before application of KCl. Maximum values of GnRH and AVP occurred 30 and 20min after KCl, respectively

With low E_2, GnRH response to KCl was lower compared with control (p=0.08) or high E_2 (p=0.001), however KCl-induced AVP release was similar between groups (p=0.6)

CONCLUSIONS

Augmentation of GnRH and AVP release by E_2 from hypothalamic slices *in vitro* reveals a direct effect of E_2 on the hypothalamus *in vivo* which may influence stress-reproduction interactions

In vitro perifusion model may be used for investigating interactions between different neurotransmitter systems in the hypothalamus

ESDAR 7th Annual Conference Dublin 4-6 September 2003

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Noradrenergic and GABA control of arginine vasopressin (AVP) release from sheep hypothalamic slices *in vitro*: sensitivity to oestradiol

SPS Ghuman[1], S Prabhakar[1], RF Smith[1] & H Dobson[1]

[1]Department of Veterinary Clinical Science, University of Liverpool, Leahurst, UK

INTRODUCTION

*Brainstem noradrenergic and hypothalamic γ-amino butyric acid neurones (GABA) modulate the stress responses of AVP in the paraventricular (PVN) nucleus *in vivo*

*Oestradiol (E_2) sensitises stress responses of the reproductive axis *in vivo.*

*Our current aim is to investigate whether E_2 influences noradrenergic and GABA control of AVP at the hypothalamic level

*This was achieved by an *in vitro* perifusion system in which hypothalamic slices were continuously exposed to media with or without E_2 (Oestradiol 17-β)

PROCEDURES

Slice collection procedures:

*At a local abattoir, brains were removed from the cranial cavity within 10min of sheep (ewe) sacrifice.

*Using skin graft blade, saggital midline hypothalamic slices (from anterior preoptic area to rostral edges of mammillary bodies, 2mm thick; 2 per sheep) were dissected and placed in glass vials containing ice-cold oxygenated perifusion media (MEM-∝; Sigma) alone or with E_2 (24pg/ml)

Perifusion procedures:

*Within 2h of collection, hypothalamic slices (per ewe distributed between groups) were transferred to 12 separate perifusion chambers (Polycarbonate syringe filter holders; Internal vol-1.5ml) perifused at 37°C with oxygenated MEM-∝ (pH7.4; Flow rate 0.15 ml/min) alone or with E_2

*After 4h equilibration, 10min fractions were collected for 4h interposed with a 10min exposure at 60min to vehicle (MEM-α), or a specific $GABA_A$ agonist (muscimol) or antagonist (bicuculline), or $GABA_B$ agonist (baclofen) or antagonist (CGP52432), or α_1-adrenoreceptor agonist (methoxamine) or antagonist (thymoxamine) at various doses (0.1-10mM; only 10mM data shown). Functional viability of slices was confirmed by exposure to a depolarising dose of KCl (100mM) for 10min in the last hour of perifusion. Concentrations of AVP were measured by radio immunoassay.

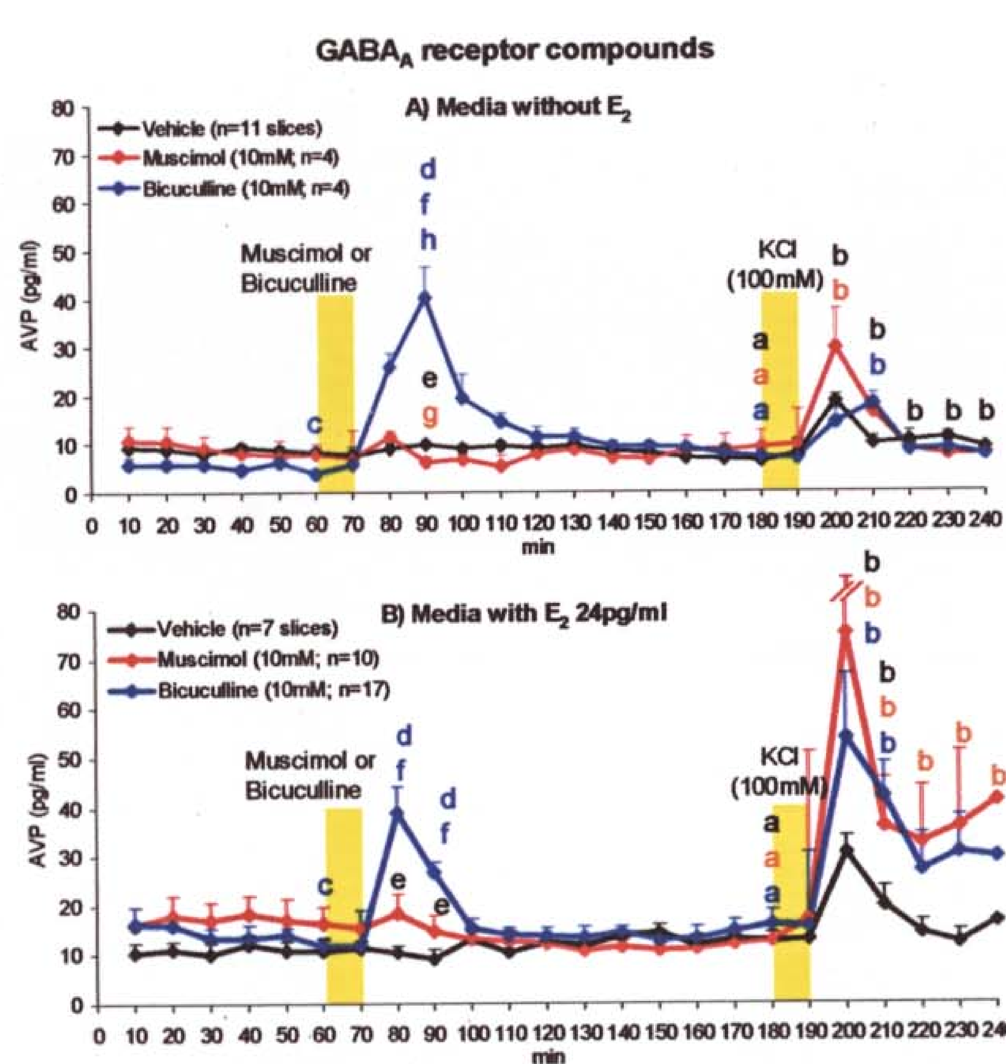


Fig 1 Effects of $GABA_A$ agonist (muscimol) or antagonist (bicuculline) and KCl challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with A) media alone or B) containing oestradiol (E_2). $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f; Tukey's pairwise comparison).

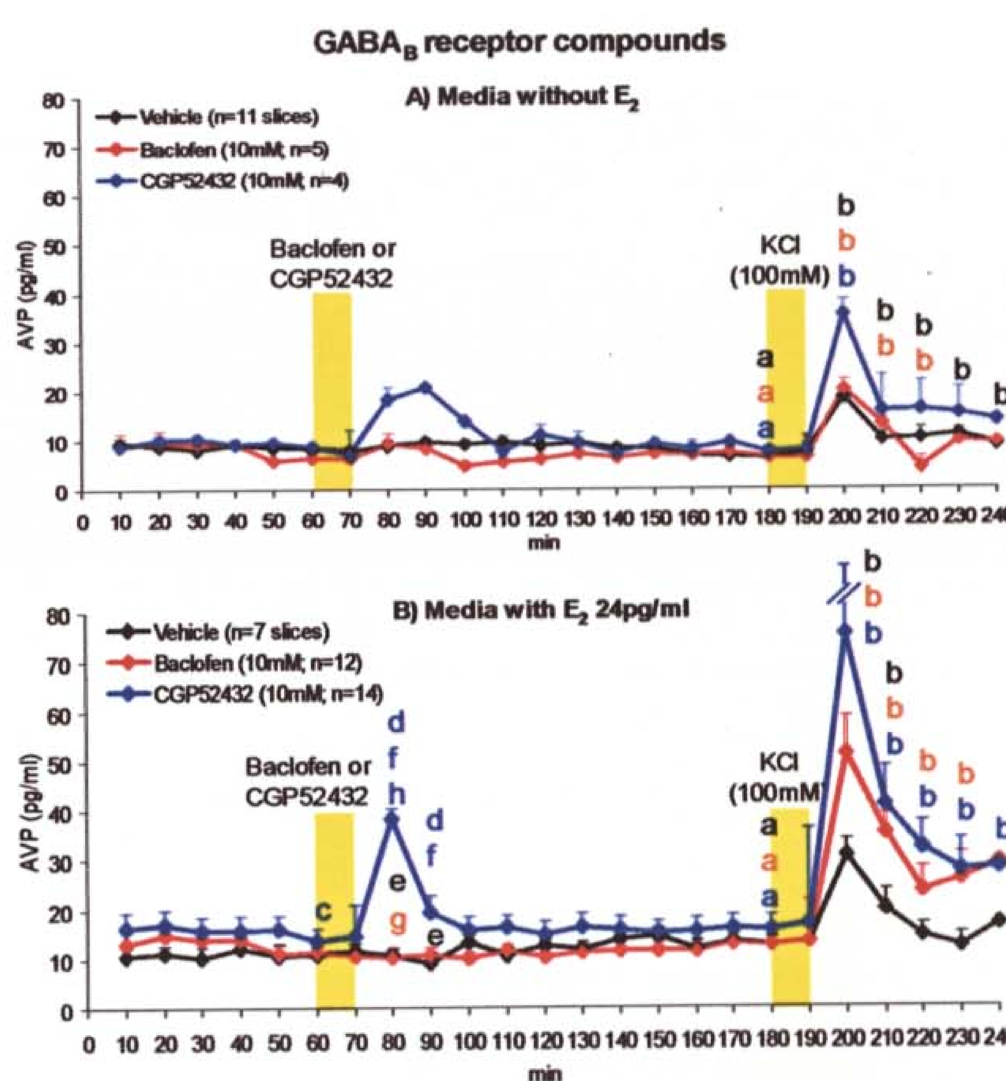


Fig 2 Effects of $GABA_B$ agonist (baclofen) or antagonist (CGP52432) and KCl challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with A) media alone or B) containing oestradiol (E_2). $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f, g *vs* h; Tukey's pairwise comparison).

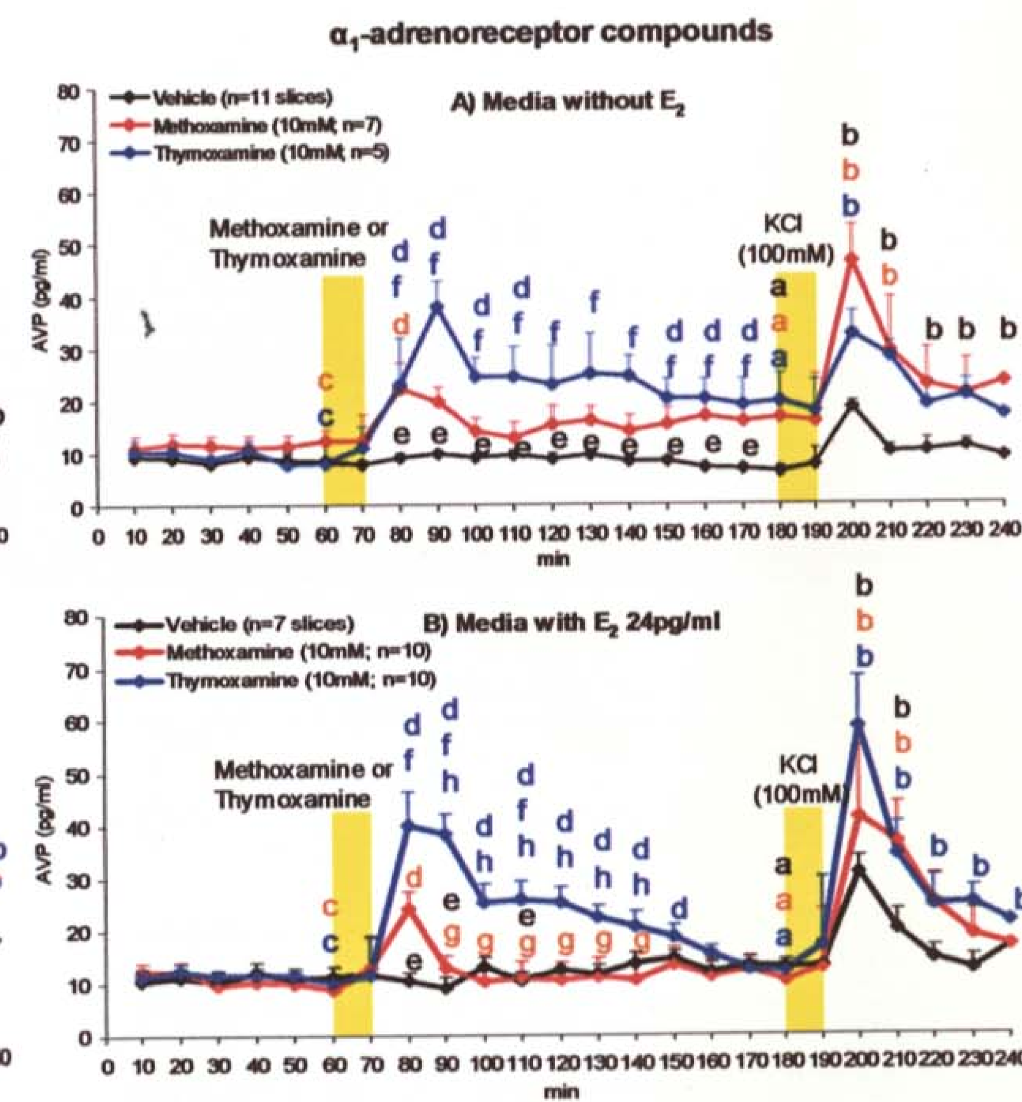
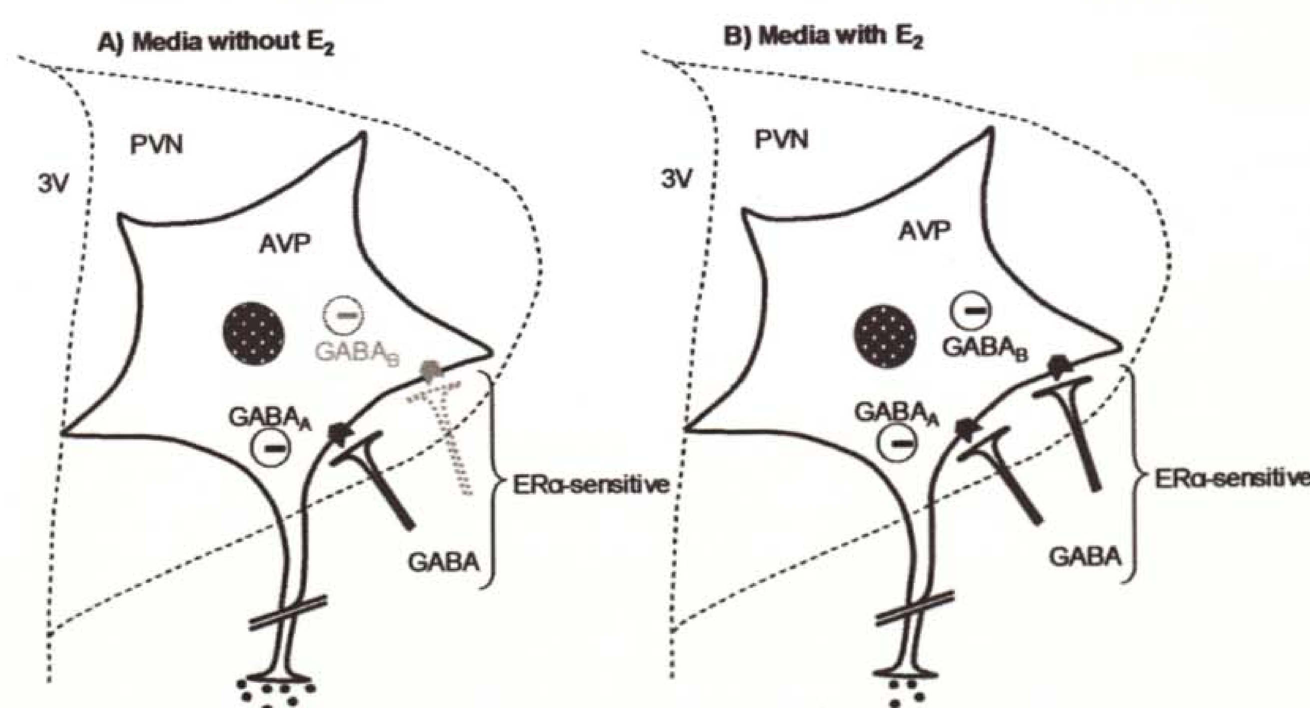


Fig 3 Effects of α_1-adrenoreceptor agonist (methoxamine) or antagonist (thymoxamine) and KCl challenge upon the release of AVP (mean±SEM) from ewe hypothalamic slices perifused with A) media alone or B) containing oestradiol (E_2). $p<0.05$; within group (a *vs* b, c *vs* d; Paired t-test), between groups (e *vs* f, g *vs* h; Tukey's pairwise comparison).

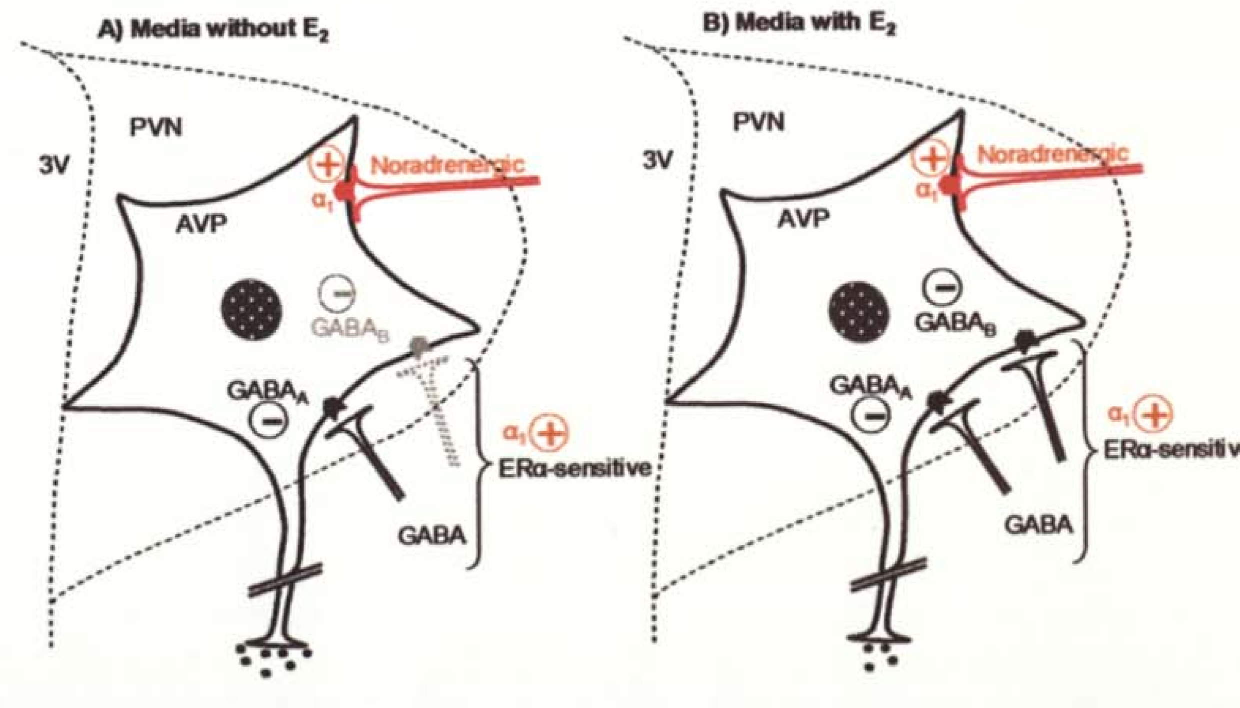
RESULTS

*Fig 1 ($GABA_A$ receptor compounds): With the antagonist, AVP release increased at 80-90min both in the presence and absence of E_2. *Fig 2 ($GABA_B$ receptor compounds): With the antagonist, AVP release increased at 80-90min only in the presence of E_2. *Fig 3 (α_1-adrenoreceptor compounds): With the agonist, AVP release occurred only at 80min both in the presence and absence of E_2. Whereas, after the antagonist, values were higher throughout the post-treatment period (80-170min) without E_2, but declined by 150min in the presence of E_2. The response to the α_1-adrenoreceptor antagonist was greater (90-140min) than the agonist only in presence of E_2.

HYPOTHESIS



Hypothesis: Influence of $GABA_A$ receptors is not modulated by the presence of E_2, however, $GABA_B$ receptors inhibit AVP neurones only in the presence of E_2. This suggests stronger inhibitory GABA influence on AVP neurones in presence of E_2. 3V, Third ventricle



Hypothesis: Final output from AVP neurones is probably dependent upon the net effect of α_1-adrenoreceptors present on AVP neurones and α_1-adrenoreceptors present on GABA neurones. In the absence of E_2 (A), α_1-adrenoreceptors present on the GABA neurones release GABA which predominantly acts through $GABA_A$ receptors, however activation of α_1-adrenoreceptors present on the AVP neurones may overcome this GABA inhibitory affect leading to net stimulatory response. But, in the presence of E_2 (B), α_1-adrenoreceptor-induced release of GABA act via both $GABA_{A\ and\ B}$ receptors which may overcome the stimulatory influence of α_1-adrenoreceptors present on the AVP neurones, ultimately leading to net inhibition of AVP neurones. 3V, Third ventricle

CONCLUSIONS

*Inhibitory influence of GABA input to AVP neurones is potentiated by E_2 through $GABA_B$ receptors *in vitro.*

*Noradrenergic control of AVP neurons through α_1-adrenoreeptors is both stimulatory and inhibitory depending upon the modulation by GABA neurones.

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Reproduction (2003) **125**, 151–163

Review

A conceptual model of the influence of stress on female reproduction

Hilary Dobson, Sarvpreet Ghuman, Sushil Prabhakar and Robert Smith

Department of Veterinary Clinical Science, University of Liverpool, Leahurst, Neston, Wirral CH64 7TE, UK

Intriguingly, similar neurotransmitters and nuclei within the hypothalamus control stress and reproduction. GnRH neurone recruitment and activity is regulated by a balance between stimulation, suppression and permissiveness controlled by noradrenaline, neuropeptide Y and serotonin from the brain stem, impact from glutamate in the medial preoptic area and neuropeptide Y in the arcuate nucleus, in opposition to the restraining influences of γ-aminobutyric acid within the medial preoptic area and opioids from the arcuate nucleus. Stress also activates neuropeptide Y perikarya in the arcuate nucleus and brain stem noradrenaline neurones. The latter project either indirectly, via the medial preoptic area, or directly to the paraventricular nucleus to release corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). Within the medial preoptic area, GnRH neurones synapse with CRH and AVP axons. Stimulation of CRH neurones in the paraventricular nucleus also activates γ-aminobutyric acid and opioid neurones in the medial preoptic area and reduces GnRH cell recruitment, thereby decreasing GnRH pulse frequency. Oestradiol enhances stress-induced noradrenaline suppression of LH pulse frequency but when applied in the paraventricular nucleus or brain stem, and not in the medial preoptic area or arcuate nucleus. The importance of CRH and AVP in the medial preoptic area needs confirming in a species other than the rat, which uses adrenal activation to time the onset of the GnRH surge. Another stress-activated pathway involves the amygdala and bed of the nucleus stria terminalis, which contain CRH neurones and accumulate γ-aminobutyric acid during stress.

Transmitting genes to the next generation is the most important activity of any organism. However, when conditions are not optimal, it is prudent to suspend reproduction until the environment is more favourable. In general, stress-induced adrenal stimulation delays or even inhibits the preovulatory GnRH–LH surge. In female rats, stress–reproduction interactions are complex because of a timing mechanism that increases adrenal gland activity before the GnRH–LH surge on the afternoon of pro-oestrus. Hence in rats, adrenal stimulation is normally associated with the facilitation of surge initiation. Therefore, comparisons with other species that do not present this complication are advantageous. The hypothalamus is larger in sheep than it is in rats and so anatomical targeting studies are more precise, and it is possible to collect frequent hypophyseal portal samples to delineate precise patterns of GnRH–LH secretion. Such techniques can be used to identify the critical link(s) between the mechanisms controlling reproduction and stress responses. For example, in the context of fertility, it is pertinent to know the importance of stress-induced changes in GnRH–LH pulsatility, oestradiol profiles and the precise timing of events leading up to the LH surge.

Many of the links between reproduction and stress-control systems involve neurotransmitters. These compounds convey messages between neurones and are crucial for the control of correct hypothalamic GnRH secretion in different reproductive phases. Individual neurotransmitters are often stimulatory (for example, noradrenaline and neuropeptide Y), whereas other neurotransmitters usually exert inhibitory influence (for example, opioids and gamma amino butyric acid (GABA)). Neurotransmitters are secreted on one side of an interneurone interface (synapse) and engage with specific receptors on the opposite neurone. Furthermore, some neurones have an auto-inhibitory system, which means that presynaptic receptors are activated so that when a neurotransmitter is released, it can act back on the secreting cell and inhibit its own production.

The present article reviews recent knowledge concerning the normal regulation of the systems controlling

Email: hdobson@liv.ac.uk

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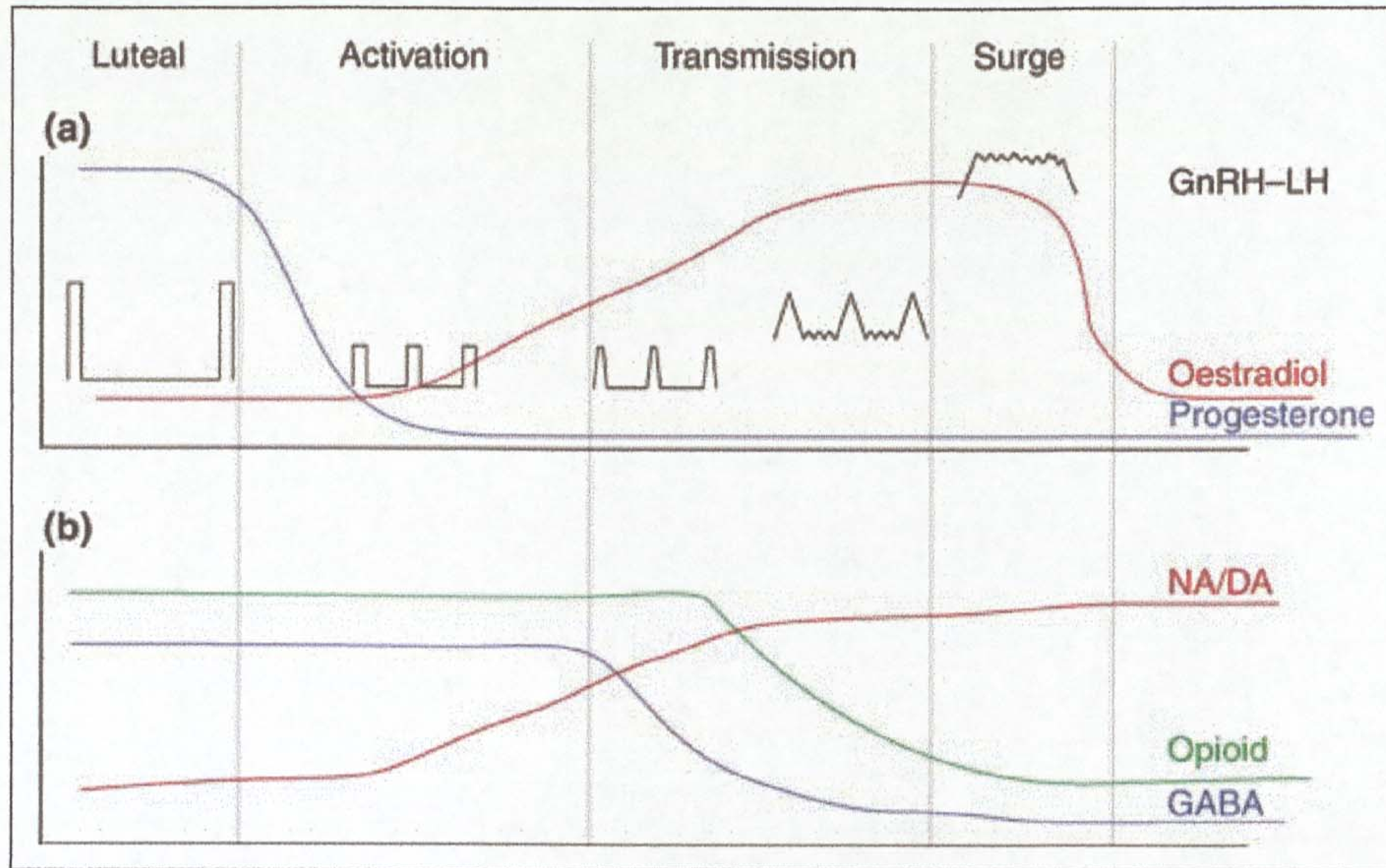

Fig. 1. (a) Luteal and oestradiol-induced activation and transmission phases before the surge secretion of GnRH (derived from Evans *et al.*, 1995, 1997) in ewes. (b) Proposed changes in neurotransmitter influence during normal GnRH secretion in ewes. DA: dopamine; GABA: gamma amino butyric acid; NA: noradrenaline.

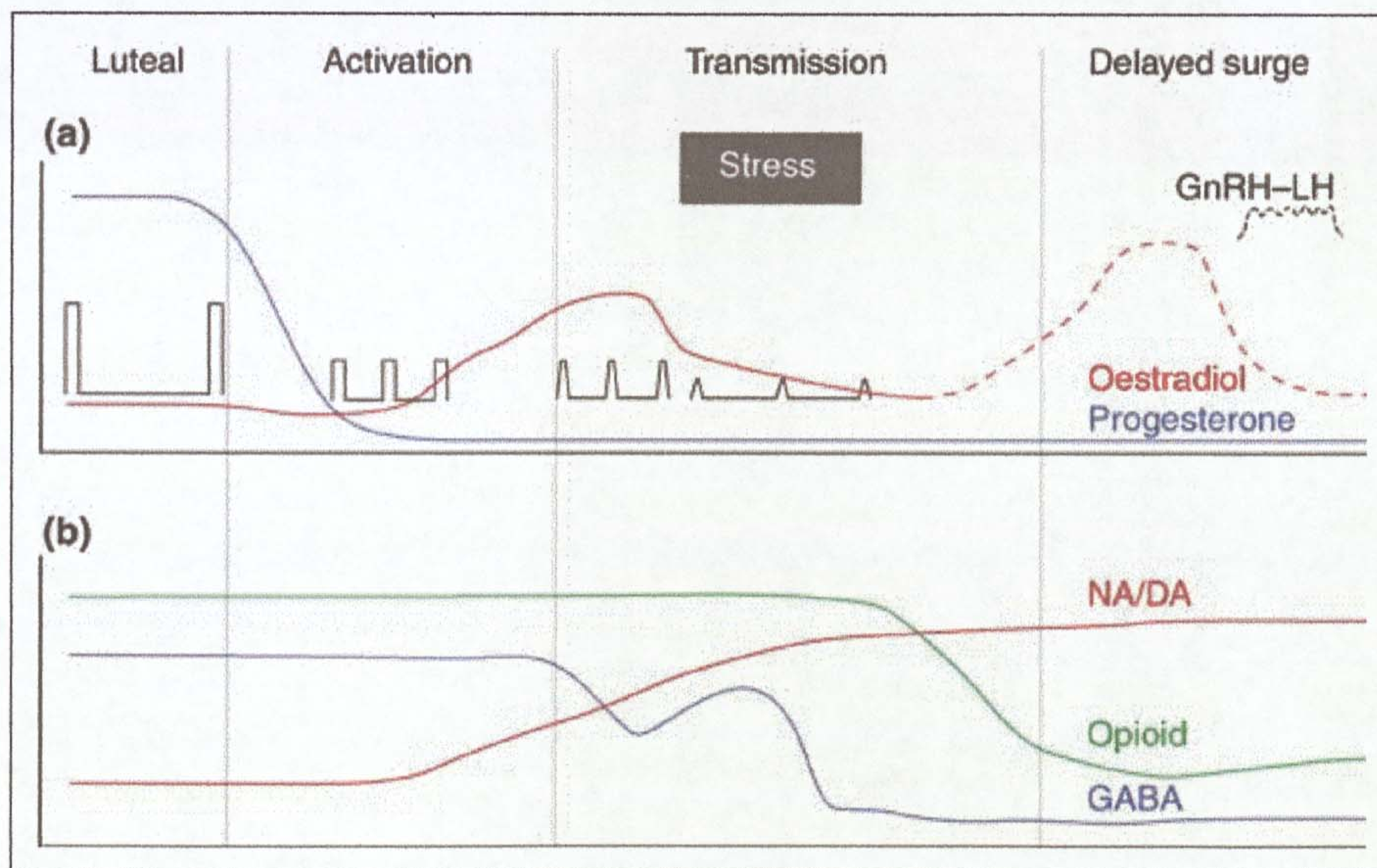

Fig. 2. (a) Changes in the endocrine milieu caused by stress in ewes (derived from Dobson and Smith, 1998, 2000). (b) Proposed changes in neurotransmitter influence during stress-induced delayed GnRH secretion in ewes. DA: dopamine; GABA: gamma amino butyric acid; NA: noradrenaline.

reproduction or stress (the hypothalamus–pituitary–ovary axis (HPO) and the hypothalamus–pituitary–adrenal gland axis (HPA)), and then examines HPO and HPA inter-relationships (in particular, the importance of oestradiol sensitivity) before placing the whole theme in the context of human and animal fertility.

Control of normal reproductive hormone profiles

Normal endocrine changes in female sheep during the follicular phase (Fig. 1) contrast with those imposed by stress (Fig. 2). The pivotal roles of different parts of the brain and hypothalamus in controlling and maintaining normal hormone profiles have been reviewed by

Herbison (1997, 1998) and Smith and Jennes (2001). Pulsatile activity is an essential intrinsic property of hypothalamic GnRH neurones and the intricate balance between GnRH synthesis and release is important to ensure that GnRH neurones are in a state of constant readiness to respond to changes in the environment (Vazquez-Martinez *et al.*, 2001). Regulation of pulsatile GnRH secretion probably involves interneurones because GnRH neurones have very few receptors for hormonal steroids. In brief, the most important site for regulation of GnRH secretion is the GnRH perikarya in the medial preoptic area of the hypothalamus (MPOA). There is still uncertainty concerning modulation of GnRH release at the other end of the axons in the median eminence (ME); similarly, debate continues over the existence of separate systems for pulsatile and surge secretion (Herbison, 1998). However, we propose that precisely controlled GnRH pulse frequency and amplitude are essential prerequisites for the timely production of appropriate GnRH–LH surge secretion. Consequently, disruption of pulsatile secretion will have deleterious effects on the production of a normal surge and will therefore reduce fertility.

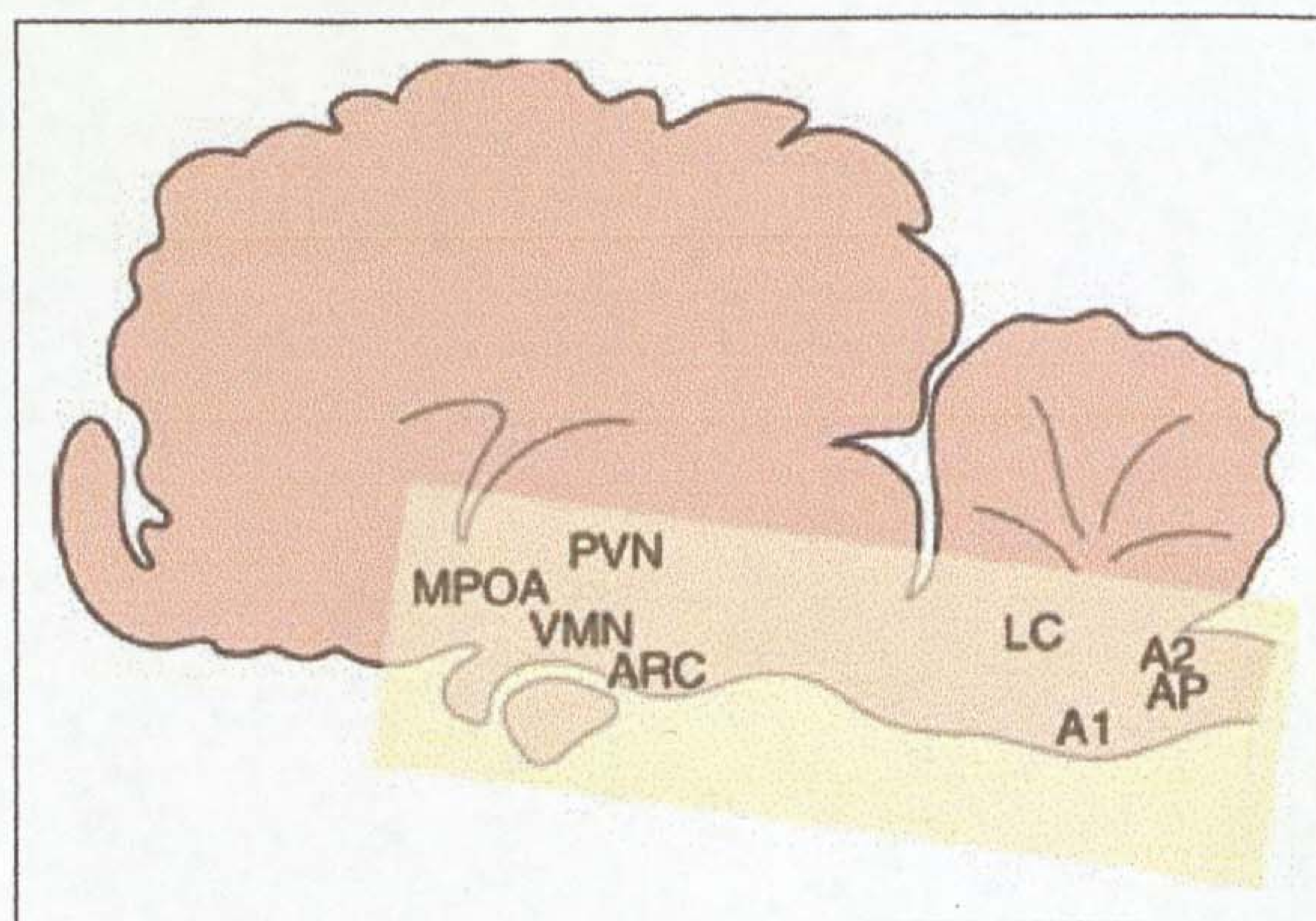

Fig. 3. Location and influence of numerous neuronal nuclei in the brain of the ewe that regulate GnRH and corticotrophin-releasing hormone (CRH)–arginine vasopressin (AVP) secretion, indicating the spatial relationship between regions of the hypothalamus (medial preoptic area (MPOA), ventral medial nucleus (VMN) and arcuate nucleus (ARC) of the medial basal hypothalamus)) and the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)). PVN: paraventricular nucleus.

Influence of the brain stem on GnRH neurones in the MPOA and modulation by oestradiol

There is a delicate balance between the positive and negative influences of oestradiol, noradrenaline and GABA in the control of GnRH perikarya in the MPOA (Figs 3 and 4). From areas within the brain stem, almost all noradrenergic cells with oestradiol receptors in regions A1 (ventrolateral medulla; VLM) or A2 (nucleus of the tractus solitarius; NTS) project axons to the MPOA to synapse with GnRH perikarya to stimulate GnRH secretion (Herbison, 1998; Scott *et al.*, 1999). Axons from noradrenaline cells in regions A1 or A2 also directly or indirectly activate GABA perikarya in the MPOA to suppress GnRH secretion. Hence, there is a balance between brain stem stimulation and suppression of GnRH secretion from the MPOA (Herbison, 1998). All GABA-ergic cells that synapse with GnRH neurones have steroid receptors and are extremely sensitive to these hormones. In the luteal phase, progesterone increases GABA tone and hence maintains very low frequency square-wave GnRH pulses (Fig. 1a). During the very early follicular phase in the presence of declining peripheral progesterone concentrations, oestradiol inhibits the autoinhibitory system of GABA cells so increased GABA concentrations are maintained in the MPOA, thereby maintaining suppression of GnRH–LH secretion (Fig. 1). Longer exposure to oestradiol during the mid-follicular phase in sheep increases noradrenaline in the MPOA culminating maximally in the late follicular phase just before the GnRH–LH surge (Robinson *et al.*, 1991). Subsequent uncoupling of noradrenaline stimulation of GABA cells follows (by an unidentified but crucial mechanism) which removes the suppression by GABA. The decrease in GABA influence and increase in noradrenaline influence lead to a surge in GnRH secretion (Herbison, 1998) and explain earlier observations in sheep that noradrenaline administered in the late follicular phase stimulates LH secretion, whereas antagonists block the LH surge (Narayana and Dobson, 1979). It must also be noted that recent studies of the MPOA using electron microscopy revealed that receptors for noradrenaline in the GnRH cell membrane are not very close to GnRH synapses in regions A1 and A2. Thus, there may be reliance on asynaptic release of noradrenaline followed by diffusion to receptors within the GnRH neurone before cell activation (Smith and Jennes, 2001). Nevertheless, the activities of GABA and GnRH perikarya in the MPOA are clearly modulated by brain stem noradrenaline neurones in response to changes in oestradiol concentration.

Influence of the medial basal hypothalamus on GnRH neurones in the MPOA and modulation by the brain stem

In addition to control by the brain stem, GnRH perikarya in the MPOA (Fig. 4) are also under the influence of afferent neurones from the arcuate nucleus (ARC) and ventromedial hypothalamic nucleus (VMN) (collectively known as the medial basal hypothalamus (MBH)). Opioid perikarya in the ARC (few of which have oestradiol receptors) project axons to the MPOA and synapse directly with GnRH cells (Goubillon *et al.*, 1999, 2002). Opioids from the ARC, noradrenaline from the brain stem and GABA neurones from within the MPOA may be involved in synchronizing the firing of different groups of neurones in the GnRH network

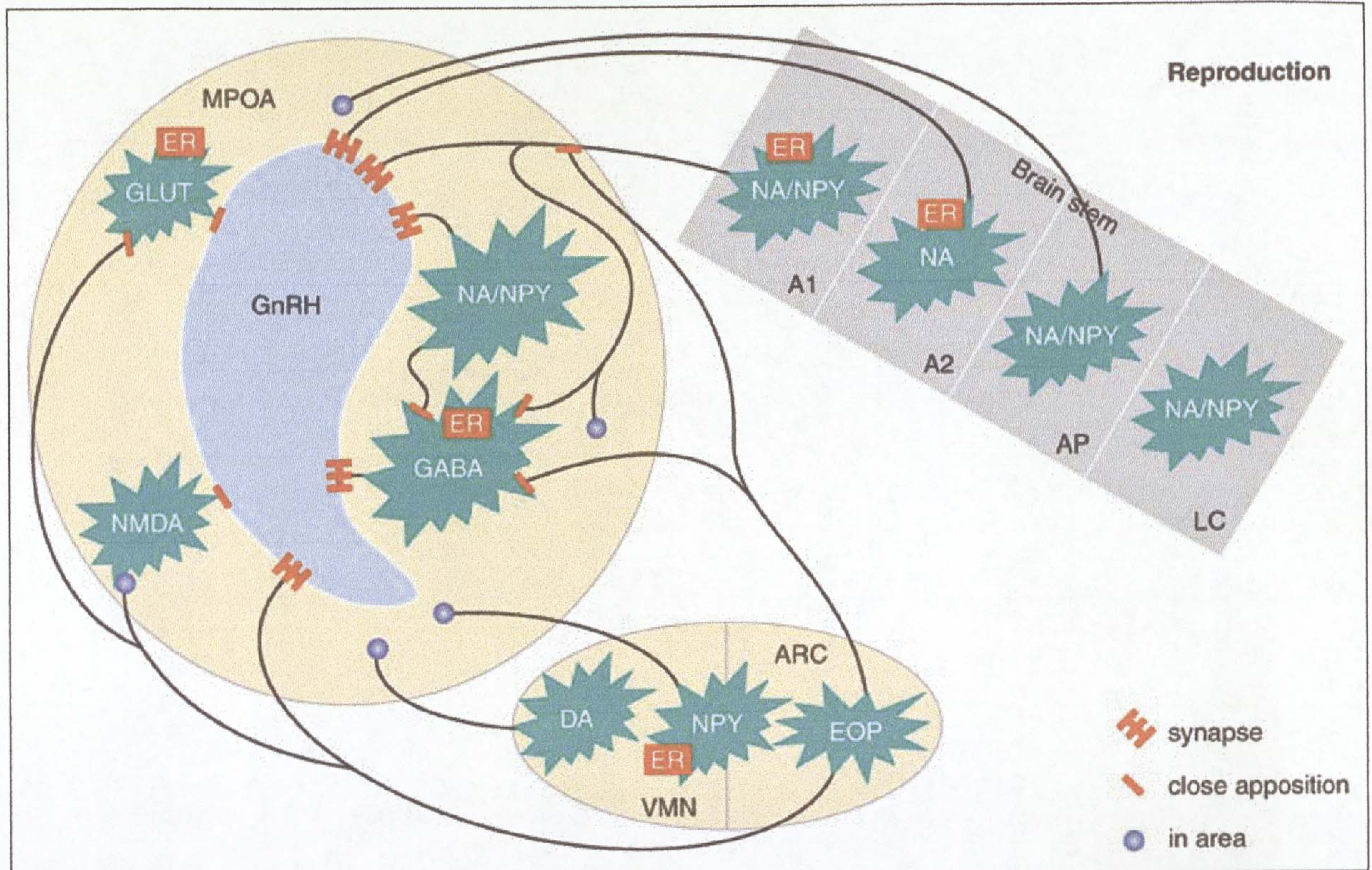

Fig. 4. Schematic representation of the location and influence of numerous neurones that regulate GnRH secretion (data amalgamated from observations in ewes and rats). The comma-shaped object represents a GnRH neurone; the grey shaded rectangular areas denote different portions of the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)) and the yellow areas indicate regions in the hypothalamus (medial preoptic area (MPOA), ventral medial nucleus (VMN) and the arcuate nucleus (ARC) of the medial basal hypothalamus). The blast shapes represent neurones containing specific neurotransmitters (noradrenaline (NA), neuropeptide Y (NPY), glutamate (GLUT), *N*-methyl-D-aspartate (NMDA), gamma amino butyric acid (GABA), endogenous opioid (EOP) and dopamine (DA)). The presence of receptors for oestradiol (ER) is indicated on appropriate neurones. The interlinking black lines between areas of the brain represent known direct axons. Identified synapses are shown by red striped bars, whereas close apposition between axons and perikarya is represented by plain red bars. If direct intercellular contact remains to be proven, the axons end in an area with a purple circle. There are axons from GnRH cells to the median eminence but these have been omitted here for clarity.

(Robinson *et al.*, 1991; Goodman *et al.*, 1995, 1996). It is not certain how many of the total of 2000 GnRH neurones discharge at any one time either for each GnRH pulse or during the preovulatory surge. However, it has been hypothesized that different interneurones alter secretion rates of various groups of GnRH axons and collaborate in the co-ordinated cascade of events that culminates in surge secretion (Karsch and Evans, 1996). Within the MPOA, opioids regulate GnRH secretion (Goodman *et al.*, 1995) but as there are few endogenous opioid (EOP) receptors on GnRH cells, other interneurones may also be involved (Thind and Goldsmith, 1988). Opioid neurones also synapse with noradrenaline interneurones in the MPOA. It is of interest that a decrease in opioid activity just before the LH surge occurs synchronously with the uncoupling of noradrenaline influence on GABA cells in the MPOA (Herbison, 1998). Thus, the MBH can modify the brain stem control of GnRH perikarya. Electrodes placed within the ARC–ME area have detected increased neuronal electrical activity after the withdrawal of progesterone in female goats. However, the frequency of electrical impulses decreases just before and during the GnRH surge (Takeuchi *et al.*, 1997).

There are other examples of MBH modulation of GnRH neurones in the MPOA. In rats, opioids restrain stimulatory catecholaminergic, glutaminergic and nitric oxide input, and enhance inhibitory GABA (Smith and Jennes, 2001). In addition, dopamine concentrations in the VMN increase just before the GnRH surge (Anderson *et al.*, 2001). Perikarya containing neuropeptide Y within the VMN–ARC also exert stimulatory influence in the MPOA (Fig. 4); neuropeptide Y is essential for GnRH surge secretion as central administration of antibodies

to neuropeptide Y blocks the LH surge in rats and sheep (Porter *et al.*, 1993). This effect could be exerted indirectly via influences on GnRH pulse frequency, or directly by interfering with any as yet unidentified 'surge mechanism'. In addition to the neurotransmitter systems already mentioned, approximately 50% GnRH cells that activate c-*fos* during the LH surge also have glutamate receptors, along with up to 80% activated GnRH cells that have *N*-methyl-D-aspartate (NMDA) receptors (Smith and Jennes, 2001). Clearly, the neuronal control of GnRH secretion involves complex but orderly interactions between the brain stem, MBH and MPOA.

Working model to explain the generation of the GnRH–LH surge

An experimental model has been proposed to facilitate closer examination of the temporal sequences leading to the GnRH–LH surge in sheep (Evans *et al.*, 1997). Here, the model is elaborated by presenting explanations in terms of the changing influence of neurotransmitters. The resultant concept indicates several possibilities, many of which are based on patchy evidence and remain to be fully tested. In the model, oestradiol implants, inserted into ovariectomized ewes 16 h after progesterone withdrawal, begin a three-stage surge induction process (Fig. 1). First, there is a signal-reading phase of 5–10 h for activation of oestradiol-sensitive neurones. Second, a signal-processing phase (from 10 to about 18 h) when message(s) are transmitted to GnRH neurones via one or more oestradiol-sensitive interneurones. A third stage involves initiation of GnRH surge release (from about 18 to 20 h). These stages approximate to the gradually merging phases of different GnRH profiles (Evans *et al.*, 1995).

Initially, there are infrequent GnRH–LH pulses (one every 4 h) in the progesterone-dominated phase that precedes the insertion of oestradiol implants. In comparison, during the follicular activation phase, pulse frequency increases and pulse amplitude decreases but square-wave GnRH pulses are discrete with barely detectable basal GnRH secretion (Fig. 1a). This pattern occurs in the presence of high GABA and opioid tone with increasing noradrenaline influence (Fig. 1b). During the early transmission phase, GABA influence declines, noradrenaline continues to increase and GnRH pulses begin to develop a more spike-like triangular shape. In the late transmission phase, within 3–4 h before surge onset, opioid influence begins to decline, GnRH pulses become more triangular and interpulse GnRH concentrations increase (Goodman *et al.*, 1995). The final surge initiation phase reveals very high GnRH concentrations and pulses are difficult to distinguish. Whether the GnRH format changes to continuous high secretion or very rapid pulses is open to debate (Evans *et al.*, 1995). Overall, it is hypothesized that GnRH neurones become progressively desynchronized and surge secretion ensues with all neurones maximally activated and secreting GnRH.

Influence of exposure to progesterone before the follicular phase

The above neuroendocrine observations might explain the balance between the effects of oestradiol and progesterone (although the following hypotheses do require further rigorous testing). In the early follicular phase of intact ewes, there is a gradual decline in progesterone after the onset of luteolysis simultaneous with a gradual increase in oestradiol stimulated from follicles by increasing LH pulse frequency. During this phase, progesterone acts via GABA neurones to hold GnRH pulse frequency in check in the face of increasing oestradiol stimulatory influence (Robinson *et al.*, 1991). Even after removal from the peripheral circulation, progesterone has long-lasting effects. The classic progesterone receptor blocker, RU486, will advance the LH surge by 9 h when administered to intact animals even 37 h after prostaglandin-induced luteolysis (surge onset at 53 h after luteolysis versus 62 h in controls; Dobson *et al.*, 2000a). Thus, progesterone modulates the influence of oestradiol.

Early oestradiol action

When considering further details of the neuronal effects of oestradiol, it is important to recognize that almost all neurones with oestradiol receptors (70% of which contain noradrenaline) in the caudal A1 region of the brain stem project to the MPOA (Fig. 4). In addition, 30% noradrenaline cells in A1 express c-*fos* (an early indicator of neuronal activity) within 2 h of oestradiol treatment (Scott *et al.*, 1999; Rawson and Clarke, 2001). In the same time frame, oestradiol also induces *c-fos* activity in approximately 20% of neuropeptide Y cells and in a few opioid cells in the VMN. However, most of the activated neurones are of unidentified phenotype (Clarke *et al.*, 2001). Thus, it is hypothesized that, under the influence of oestradiol during the early activation phase, noradrenaline neurones from the brain stem and from within the MPOA initially activate MPOA GABA perikarya. This activation of GABA neurones by noradrenaline, along with the neuropeptide Y and opioid influence of MBH origin, decrease pulse amplitude and increase pulse frequency (Scott *et al.*, 1992; Smith and Jennes, 2001). It is proposed that, during the transmission phase, GnRH pulses begin to change shape as the effects of GABA gradually diminish (Fig. 1). In the late transmission phase, a decrease in opioid tone further prolongs pulse duration and increases GnRH secretion between pulses (Goodman *et al.*, 1995; Evans *et al.*, 1997). Eventually, at the onset of the surge, all inhibition is removed.

Clearly, GnRH secretion is regulated by a balance between stimulation, suppression and permissiveness controlled by noradrenaline from the brain stem, sudden impact from glutamate in the MPOA and neuropeptide Y from the ARC, in opposition to the inhibitory influences of GABA within the MPOA and opioids from the ARC. Furthermore, it is pertinent at this point to note that oestradiol must be administered into the VMN rather than the MPOA to generate an LH surge (Herbison, 1998).

Other roles of oestradiol in generation of the GnRH–LH surge

Removal of oestradiol implants before initiation of the surge (that is, after only 10 h exposure) influences neither the timing of surge onset nor the amplitude of the GnRH surge. However, less LH is released, indicating that there is a requirement for longer oestradiol priming at the pituitary gland for a full amplitude LH surge (Evans *et al.*, 1997). Oestradiol enhances hypothalamic GnRH synthesis and pituitary responsiveness to GnRH by increasing GnRH receptor mRNA, and thus the number of receptors, in gonadotrophes, creating greater stores of LH (Hamernik, 1995; Brooks and McNeilly, 1996; Jimenez-Linman and Rubin, 2001).

In summary, oestradiol has marked effects at the hypothalamus and the pituitary gland to produce regulated GnRH pulses and a normal, appropriately timed LH surge.

Normal HPA endocrine responses to stressors

Physiological amounts of insulin are required for normal neuronal activity but the sudden exposure to bolus insulin injections will activate the HPA axis, as will many other stressors, for example, transport, restraint or isolation. As part of this response, ACTH release from the pituitary gland is stimulated by activation of perikarya in the hypothalamic paraventricular nucleus (PVN; Figs 3 and 5). The two releasing factors from the PVN, corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP), are secreted from the ME into the hypophyseal portal system to release ACTH from the pituitary gland but the relative importance of CRH and AVP is currently controversial (Smith *et al.*, in press). More CRH than AVP is released in rats, whereas the reverse occurs in sheep. The CRH : AVP ratio may be governed by the intensity of the stressor, with CRH predominating after low doses of insulin whereas more AVP is secreted after high doses (Caraty *et al.*, 1990). However, CRH is a more potent stimulator of ACTH secretion than is AVP in sheep (McFarlane *et al.*, 1995). Nevertheless, AVP is required in stress-induced suppression of reproductive activity, as revealed by the ability of AVP antagonists to prevent the suppressive effects of insulin on LH secretion (Heisler *et al.*, 1994).

Influence of the brain stem and MPOA on PVN secretory activity

Higher up the system (Fig. 5), stressors increase the firing rate of regions in the brain stem, particularly noradrenaline–neuropeptide Y neurones in the A1 (VLM), A2 (NTS) and A6 (locus coeruleus; LC) regions. There are synaptic contacts between noradrenaline–neuropeptide Y nerve fibres and CRH perikarya in the PVN, although there have been no confirmatory retrograde or anterograde double-labelled studies to confirm the location of the perikarya of these axons (Krysiak *et al.*, 1999). In contrast, detailed tracing studies in rats have shown that noradrenaline perikarya send axons from the A1 (VLM) to the MPOA that, in turn, synapse with afferent neurones projecting to the PVN, and that these axons are activated during stress (Kawano and Masuko, 1999, 2000). It is important to establish the significance of CRH pathway interactions through the MPOA in species other than the rat that do not have an increase in corticoids associated with the GnRH–LH surge.

Involvement of receptors during negative feedback

A stimulatory role of noradrenaline in the HPA is confirmed by the sustained release of ACTH after prolonged central noradrenaline administration, in contrast to the short-lived ACTH secretion that occurs during exposure to spontaneous stressors. One explanation of these contrasting scenarios could involve immediate activation of a negative feedback system exerted by glucocorticoid receptors on CRH cells in the PVN (Fig. 5). In addition, the hippocampus contains many mineralocorticoid and glucocorticoid receptors and, although there is no direct innervation to the PVN, there are projections from the hippocampus to the ARC and VMN that exert negative feedback on PVN activity. Furthermore, axons from neuropeptide Y perikarya with glucocorticoid receptors in the ARC project to the PVN. It is reasonable that there should be an efficient negative feedback system in view of the deleterious effects of overstimulation of the HPA, that is, prolongation of enhanced glucose metabolism, altered vascular dynamics and deranged immune responses.

Other pathways modulating PVN secretion

In addition to a significant role in emotional behaviour and motivation, the rat central nucleus of the amygdala (CeA) also contains many CRH perikarya with efferent pathways to the hypothalamus through the lateral bed nucleus of the stria terminalis (BNST) and onward to the MPOA. Lesions of these tracts lower stress-induced CRH concentrations in the ME (Tellam *et al.*, 2000).

It has been suggested that CRH is a neurotransmitter as well as a neurohormone (Tellam *et al.*, 2000). CRH injected intracerebroventricularly (i.c.v.) or directly into

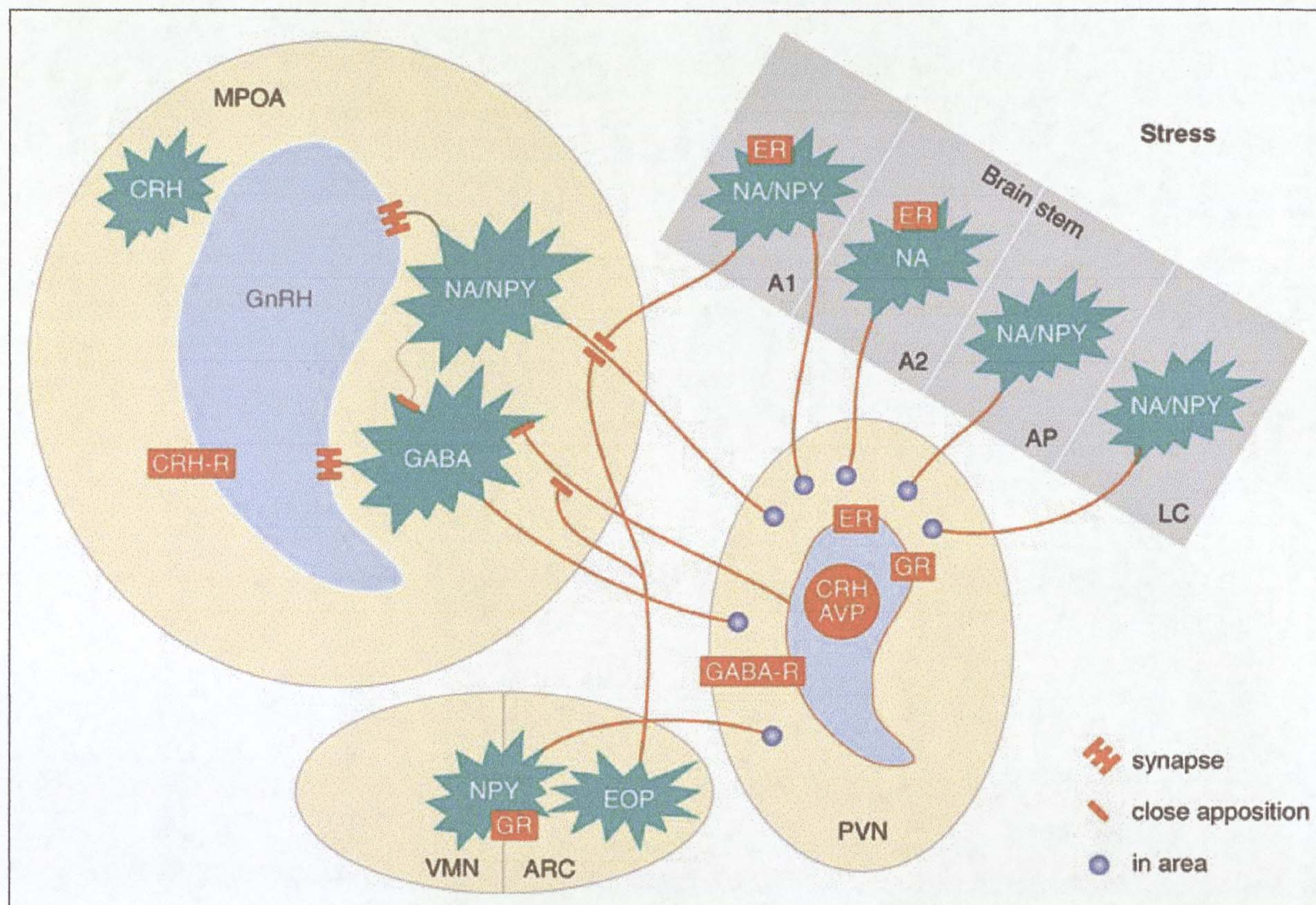

Fig. 5. Schematic representation of the location and influence of numerous neurones that regulate corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) secretion from the paraventricular nucleus (PVN) (data amalgamated from observations in ewes and rats). The comma-shaped object represents CRH–AVP or GnRH neurones; the grey shaded rectangular areas denote different portions of the brain stem (A1 and A2 regions, the area postrema (AP) and the locus coeruleus (LC)) and the yellow areas indicate regions in the hypothalamus (medial preoptic area (MPOA), ventral medial nucleus (VMN) and the arcuate nucleus (ARC) of the medial basal hypothalamus). The blast shapes represent neurones containing specific neurotransmitters (noradrenaline (NA), neuropeptide Y (NPY), glutamate (GLUT), *N*-methyl-D-aspartate (NMDA), gamma amino butyric acid (GABA), endogenous opioid (EOP) and dopamine (DA)). The presence of receptors for oestradiol (ER) is indicated on appropriate neurones. The presence of receptors for CRH, gamma amino butyric acid (GABA) or glucocorticoids (CRH-R, GABA-R or GR) are indicated on appropriate neurones. The interlinking red lines between areas of the brain represent known direct axons. There are axons from GnRH and CRH–AVP cells to the median eminence but these have been omitted here for clarity.

the LC increases the neuronal firing rate in this region and increases the release of noradrenaline in areas receiving projections from the LC (Fig. 5). Indeed, there is evidence to show that, in response to a variety of stressors, there is a cascade relationship between noradrenaline and CRH secretion in all areas containing CRH perikarya, that is the LC, CeA, BNST, MPOA and PVN (Koob, 1999). Thus, CRH, as a neurotransmitter, may enhance activation of the HPA as well as being involved in final ACTH output.

Reproductive hormone profiles during disruption by stressors

After presenting individual HPO and HPA control mechanisms, changes in reproductive hormone profiles during exposure to stress will be examined, and this is followed by a discussion of the interactions involved within the brain.

Effects of stressors on tonic GnRH–LH pulsatile secretion

There are now many examples of gonadectomized animals (male and female) exhibiting a reduction in GnRH and hence LH pulsatility within 5–10 min of exposure to stress (Fig. 2a) indicating non-genomic effects on membrane polarization of interneurones (Fig. 2). It is difficult to ascertain from the available literature whether there are differences between stressors in their effects on pulse frequency or amplitude; if frequency of GnRH pulses is reduced, this may indirectly affect

amplitude because basal values will differ. The use by different laboratories of different steroid treatments before exposure to stress also complicates the issue. Furthermore, most studies have used LH measurements to reflect changes in GnRH, but it is now known that not all GnRH pulses are accompanied by LH secretion. However, changes in frequency do indicate a major effect at the hypothalamus. Indeed, the frequency, but not the amplitude or duration, of hypothalamic multi-unit electrical activity is decreased in stressed goats compared with controls (Takeuchi *et al.*, 1997).

In rats, adrenaline modulates LH frequency, whereas noradrenaline affects both frequency and amplitude (Smith and Jennes, 2001). By comparison, in non-stressed sheep, GABA neurones control pulse frequency, whereas noradrenaline and opioid neurones mediate changes in pulse amplitude (Scott and Clarke, 1993; Goodman *et al.*, 1995, 1996). As CRH predominates during 'weak' stressors and additional AVP is secreted during more intense stress (Caraty *et al.*, 1990), it is possible that CRH affects pulse frequency via GABA–noradrenaline neurones, whereas AVP responses involve opioidergic cells, particularly as, in rats, i.c.v. AVP affects pulse amplitude but not frequency (Cates *et al.*, 1999). This speculation urgently requires testing under closely controlled conditions by measuring GnRH in portal blood and by histochemical examination of multi-labelled tissues.

Effects of stress on GnRH–LH surge secretion

Acute stressors can also interrupt the occurrence of a normal GnRH–LH surge during spontaneous follicular phases in intact ewes and in steroid-treated ovariectomized animals (Fig. 2a). After exposure to the severe stressors of endotoxin or high dose insulin during the activation phase, the onset of the LH surge is delayed by approximately 10 h. Later, during the transmission phase, endotoxin has no effect on timing or other surge parameters. However, the onset is delayed by insulin and surge maximum concentrations are lower. Even the lesser stress of transport delays and reduces the magnitude of the LH surge when imposed during the late transmission or surge onset periods (Smith *et al.*, in press).

The influence of stressors at the pituitary gland has been revealed by the reduced secretion of LH in response to GnRH, both *in vivo* and *in vitro* (Phogat *et al.*, 1999), and could be one explanation for reduced maximum surge values. Reduced surge concentrations probably relate to the importance of pulsatile GnRH in maintaining the pituitary gland in a primed state. In addition, continued oestradiol exposure is curtailed during ACTH-induced reduction in LH pulsatility in the follicular phase, and this will have consequences at both the hypothalamus and pituitary gland (Dobson *et al.*, 2000b).

Mechanisms of interactions between HPA and HPO within the brain

Stress-induced changes in reproductive hormone profiles are the result of altered neurotransmitter activities mediated by different neurones in several locations.

Mediation by CRH and AVP

As the PVN is activated in all stressful situations, both CRH and AVP are prime candidates as mediators of reduced GnRH secretion. Indeed, CRH receptors have been identified on GnRH perikarya in rat immortal cell lines (Tellam *et al.*, 2000; Fig. 5). Furthermore, administration of CRH or AVP either i.c.v. or directly into the MPOA (but not the PVN, ARC or ME) reduces GnRH secretion in rats or monkeys, and reversal of the effect by naloxone indicates opioid involvement (for reviews, see Rivier and Rivest, 1991; Phogat *et al.*, 1997). Reversal of the CRH–AVP-induced suppression of GnRH by prior oestradiol treatment in monkeys indicates that the influence of steroids is balanced in different ways in different species, or that there is an oestradiol dose effect (Chen *et al.*, 1992).

Confusingly, in sheep, i.c.v. CRH administration or exposure to transport can increase GnRH–LH output, but only during periods of oestradiol negative feedback (Caraty *et al.*, 1997; Dobson *et al.*, 1999). This finding could be the result of neurotransmitter action unconnected to the stress axis, or steroid feedback mechanisms may be important. As a further complication, restraint of CRH knockout mice also decreases pulsatile and surge LH secretion indicating the presence of an opioid or AVP compensation mechanism. The importance of an AVP mechanism is emphasized by the fact that insulin has no effect on LH pulsatility in Brattleborough rats (a strain in which AVP is absent). More evidence lies in the inability of the antagonist helical CRH always to reverse the effects of CRH on LH release (Jeong *et al.*, 1999). Most of these observations can be explained by a co-operative compensatory system between CRH and AVP. One further major factor to be taken into account is that destruction of the PVN does not obliterate the suppression of LH pulsatility during stress (Rivier and Rivest, 1991). Therefore, effects in rats must be mediated at least in part by pathways other than via the PVN, for example, via the CeA or the BNST (Koob, 1999).

Interactions between the MPOA, PVN and the brain stem

Several neuronal interactions, direct or indirect, have been recognized between the MPOA and the PVN (Fig. 5). There are CRH perikarya in the MPOA and synapses occur in the MPOA between GnRH neurones and CRH-positive axons and AVP-positive axons, both of

unidentified origin in rats, monkeys and goats (MacLusky *et al.*, 1988; Thind and Goldsmith, 1988; Kikusui *et al.*, 1997). In addition, CRH neurones from the PVN project to GABA cells in the MPOA (Cullinan, 2000). Thus, indirect mechanisms operate by provoking activity in the PVN and hence increasing delivery of CRH to the MPOA. For example, the PVN receives neuronal inputs from many nuclei (Fig. 5): noradrenaline–neuropeptide Y projections containing oestradiol receptors from the regions A1 and A2; dopamine neurones from the area postrema (part of the VLM activated by glucose availability and other stressors); GABA neurones from the MPOA; and neuropeptide Y axons from the ARC. Indirectly, catecholaminergic neurones from the A1 region synapse in the MPOA with neuropeptide Y–noradrenaline afferent neurones projecting to the PVN (Kawano and Masuko, 1999, 2000).

The significance of the increased PVN influence on MPOA activity is revealed by the stress-induced concurrent activation of CRH neurones in the PVN and the reduction in the number of GnRH cells expressing *c-fos* within the MPOA, both during the period when GnRH–LH secretion declines (Briski and Sylvester, 1998). Most of the above observations are from rats and, again, it is imperative to have confirmation in another species that does not positively associate increased adrenal activity with the onset of the GnRH–LH surge.

Mediation by suppressive opioids and GABA, or stimulatory neuropeptide Y and serotonin

The influence of opioid mediation in stress-induced GnRH suppression is revealed by the ability of naloxone to reverse the decrease in pulse frequency after restraint, injection of 2-deoxy-glucose (2DG; to rapidly lower glucose availability) or insulin administration in sheep or rats, but not in monkeys (Clarke *et al.*, 1990; Chen *et al.*, 1992; Cagampang *et al.*, 1997). Insulin-induced delays in the LH surge in sheep are also reversed by naloxone, whereas the progesterone receptor antagonist, RU486, is unable to bring forward the delayed LH surge (Dobson and Smith, 2000). As there is an increase in mRNA encoding pro-opiomelanocortin (POMC) in the ARC after glucoprivation in rats, all these effects are probably mediated by opioid influence from the ARC via synapses in the MPOA. These opioid synapses occur both with noradrenaline–neuropeptide Y projections from the MPOA to the PVN, and with CRH axons from the PVN to the MPOA (Kawano and Masuko, 1999, 2000). Local opioid antagonists are effective in reversing decreased GnRH–LH pulsatility only when applied in the MPOA and not the ARC, emphasizing that it is the MPOA that is the actual site of action. Furthermore, naloxone also reverses the decline in the number of GnRH neurones recruited in the MPOA after treatment of oestradiol-sensitized ovariectomized rats with 2DG (Briski and Sylvester, 1998). The opioid-induced low recruitment of GnRH neurones during stress probably interferes with synchronized firing of GnRH neurones, leading to disrupted LH secretion (Goodman *et al.*, 1995).

Suppressive GABA neurones have been implicated in stress-induced reductions in GnRH secretion. During chronic stress, concentrations of mRNA encoding GABA increase in the MPOA and BNST. Apart from a direct effect on GnRH neurones, as part of a negative feedback system, high GABA concentrations could exert influence via the above mentioned neuronal pathways to downregulate CRH–AVP activity in the PVN (Fig. 5). There are GABA receptors on 95% of CRH neurones in the PVN (Cullinan, 2000). Furthermore, GABA injected directly into the PVN or MBH decreases CRH and, conversely, GABA antagonists administered into the PVN increase CRH–AVP (Cole and Sawchenko, 2002). Indeed, insulin-induced effects on LH pulse frequency and amplitude are reversed in monkeys by the $GABA_A$ receptor antagonist, alprazolam (van Vugt *et al.*, 1997). Therefore, it is clear the GABA is involved in the suppression of LH secretion (Fig. 2b) but future work is needed to examine more closely the simultaneous effects of stressors on GABA and GnRH neurone activity.

Stimulatory neuropeptide Y may have an indirect effect during stress-induced reductions in LH pulsatility. Insulin treatment increases neuropeptide Y in the ARC and PVN (Fig. 5), and administration of neuropeptide Y stimulates CRH release thus increasing peripheral opioid, ACTH and corticoid secretion (Porter *et al.*, 1993; Krysiak *et al.*, 1999). Furthermore, the decline in LH pulsatility after neuropeptide Y administration in rats is regulated by the prevailing steroid milieu, possibly as a result of oestradiol-mediated increased opioid influence. There are neuropeptide Y perikarya in the ARC with projections to the PVN (Fig. 5), but it has not been established whether they are part of the 15% of the neuropeptide Y population with oestradiol receptors (Clarke *et al.*, 2001). However, in the breeding season of sheep, administration of neuropeptide Y into the third ventricle has no effect on LH pulsatility in the presence or absence of oestradiol, although peripheral concentrations of cortisol increase (Porter *et al.*, 1993). Further studies are required to establish the precise role of neuropeptide Y in stress-induced reductions of GnRH–LH secretion.

With respect to serotonin, the possible involvement of the Raphe nuclei in the brain stem is revealed by the existence of CRH neurones that innervate this area where there are also CRH receptors. Administration of CRH inhibits serotonin release and Raphe neuronal activity (Price *et al.*, 1998). Serotonergic axons from the Raphe nuclei also project into the MPOA to enhance GnRH secretion (Smith and Jennes, 2001), and so the MPOA could be influenced by the stress-induced reduction in serotonin activity.

Influence of oestradiol on stress-induced suppression of GnRH–LH secretion

The effects of stressors on GnRH–LH secretion are intensified by gonadal steroids (oestradiol in females and testosterone in males), although conversion of testosterone to oestradiol is likely in males (Maeda *et al.*, 1994; Adam and Findlay, 1998; Tilbrook *et al.*, 2000). The sex steroids modify CRH transcription and expression (Tellam *et al.*, 2000), and CRH neurones have been identified in the MPOA in different species (see above). Indeed, in a series of elegant experiments, K-I. Maeda's group showed that oestradiol treatment sensitizes LH secretion to suppression by stressors (glucoprivation, fasting or immobilization) at doses that do not change the characteristic LH pulsatility pattern in ovariectomized rats. Catecholamines increase the number of oestradiol receptors in the hypothalamus, and this has implications for the interaction between stress and GnRH–LH suppression. Stress-induced noradrenaline suppression of LH pulse frequency is enhanced by oestradiol, but only by localized action in the PVN or A1–A2 regions and not in the MPOA or ARC (Fig. 4; Maeda *et al.*, 1994; Nagatani *et al.*, 1996; Cagampang *et al.*, 1997). Indeed, during stress, the number of noradrenaline–dopamine cells with oestradiol receptors increases in the PVN and brainstem (A1 and A2 regions) but not in the MPOA, ARC or VMN (Estacio *et al.*, 1996; Reyes *et al.*, 2001). Furthermore, α-helical CRH will reverse the effects of stressors on LH pulse frequency and amplitude in oestradiol-sensitized rats, whereas i.c.v. AVP only reduces LH pulse amplitude (Cates *et al.*, 1999). There is an oestrogen response element on the mRNA encoding CRH and an increase in this CRH mRNA occurs in the PVN after oestradiol treatment late in pro-oestrus (Herbison, 1998). Thus, there is a distinct modulating effect of oestradiol on neurotransmitter activity during stress. Again, observations must be interpreted with care in rats because of the corticoid-induced LH surge late in pro-oestrus in this species.

Other aspects of HPO and HPA interactions

Although GnRH cell bodies are most abundant in the MPOA in rats, a small population of uncertain function has been identified in the ARC in sheep. The efferent axons of both MPOA and ARC populations project into the ME close to the hypophyseal portal blood capillaries. There are receptors for noradrenaline, glutamine or neuropeptide Y at the efferent axon–capillary junction but it is thought that these neurotransmitters have minimal pre-synaptic control of GnRH release in the ME (Smith and Jennes, 2001). Furthermore, CRH neurones are not associated with GnRH rich areas in the ME. However, it is possible that changes occur in glial cell ensheathment and tanycyte architecture. These cells encasing GnRH neurones might prevent axon terminals temporarily forming synapses with the portal capillaries. Equally, glial changes in the ARC and MPOA are associated with regulation of GnRH release. The presence of oestradiol receptors and structural changes within 5 h of oestradiol treatment indicate an involvement in reproduction but stress-associated changes still require elucidation (Herbison, 1998; Viguie *et al.*, 2001).

All stressors result in increases in corticoids and catecholamines from the adrenal gland medulla but it is unlikely that these have a major influence on LH secretion because stress effects on GnRH have been observed in adrenalectomized animals (Rivier and Rivest, 1991). Furthermore, peripheral catecholamines or cortisol may not cross the blood–brain barrier (Deaver and Dailey, 1982; Adam and Findlay, 1998). Nevertheless, prolactin and cortisol have been associated with suppression of transcription of the GnRH promoter in rats (Tellam *et al.*, 2000). In sheep, prolonged high-dose cortisol infusions interfere with LH pulses and surge secretion but, confusingly, inhibition of cortisol synthesis by metyrapone does not inhibit endotoxin-induced suppression of LH release (MacFarlane *et al.*, 2000; Karsch *et al.*, 2002).

Conclusions

In normal intact animals, oestradiol activation of GnRH–LH surge secretion involves an initial stimulatory oestradiol signal but restraining modulation is mediated by noradrenaline and opioid regulation of suppressive GABA neurones. During the later transmission phase in which oestradiol concentrations are higher, a gradual removal of opioid influence may mediate the uncoupling of noradrenaline restraint on GABA cells, resulting in a (now oestradiol-independent) more positive noradrenaline influence on GnRH–LH release. The concomitant overall increase in GnRH secretion, along with further increased oestradiol, enhances synthesis of GnRH receptors and LHβ in the pituitary gland. At the end of the transmission phase, neurotransmitters control the recruitment of more GnRH neurones, culminating in co-ordinated hyperpolarization and massive outpouring of GnRH into the portal capillaries to cause secretion of the prepared stores of LH. Depending on the intensity, stress impedes the activation or transmission phases, or both, by stimulation of oestradiol-sensitive PVN activity that interferes with GnRH neurone recruitment, and hence GnRH pulsatility decreases and surge secretion is disrupted. Several reproductive situations involve suppression of GnRH–LH pulsatility mediated by increased sensitivity to oestradiol in different parts of the hypothalamus and brain stem. For example, seasonality involves the A14 and A15 regions, whereas undernutrition, fasting or other stressors involve the area postrema of the A1 region of the brain stem in collaboration with the PVN and possibly the CeA.

Therefore, in terms of human and animal fertility, how important are changes GnRH–LH pulse frequency and amplitude, oestradiol profiles and the precise timing of events leading up to the LH surge? During maturation, gametes are closely associated with steroid-producing support cells (Leydig cells in the testis or ovarian follicular granulosa cells). Interruption of LH pulsatility (by GnRH antagonists or by stressors) in the follicular phase lowers oestradiol production and reduces the subsequent rate of blastocyst formation (Dobson and Smith, 1998; Oussaid *et al.*, 1999). Oestradiol is also required for expression of behavioural oestrus, along with increased GnRH concentrations in the hypothalamus (Caraty *et al.*, 2002). Furthermore, increased CRH has behavioural effects that appear to be independent of the pituitary–adrenal axis, for example, i.c.v. administration of CRH decreases food intake and sexual behaviour in oestrogen-primed rats (Koob, 1999).

Delayed LH surges and the resulting extended follicular phases are deleterious to fertility (Dobson and Smith, 2000). Indeed, cows with 'production diseases' such as hypocalcaemia, dystocia, ketosis or lameness are less fertile, possessing smaller follicles than healthy herd-mates in the breeding period approximately 6 weeks after calving (W. J. Clarke, H. Dobson and R. F. Smith, unpublished). These smaller follicles could be the result of lowered LH pulse frequency or stress-mediated interference with gonadotrophin recruitment of early follicles. Similar studies on human patients with anxiety driven subfertility would be of interest.

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Stress and the control of LH secretion in the ewe

R. F. Smith[1], S. P. S. Ghuman[1], N. P. Evans[2], F. J. Karsch[3] and H. Dobson[1]

[1]Department of Veterinary Clinical Science, University of Liverpool, Leahurst, Neston, Cheshire CH64 7TE, UK; [2]Department of Veterinary Preclinical Studies, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK; and [3]Reproductive Sciences Program, Department of Physiology, University of Michigan, Ann Arbor, MI 48109, USA

Stress influences the activity of the reproductive system at several sites. One of the most significant effects is at level of the GnRH secretory system to reduce GnRH pulsatility and thus LH pulsatility. This in turn reduces the oestradiol signal that stimulates the GnRH–LH surge in the follicular phase. Three sequential phases have been identified in the induction of the GnRH–LH surge by oestradiol: (i) activation, (ii) transmission and (iii) surge secretion. There is evidence that administration of endotoxin prevents activation but not transmission, hypoglycaemia blocks both activation and transmission, whereas truck transport is effective during the late, but not early, transmission phase. Opioids mediate the suppressive effects of hypoglycaemia on both LH pulsatility and the delayed onset of the LH surge in ewes. The exact neurocircuitry used in sheep is yet to be identified but many of the connections that are proposed as important in rats are present in sheep. Corticotrophin-releasing hormone (CRH) neurones in the paraventricular nucleus that project axons to the median eminence probably do not directly inhibit GnRH, but either afferent or parallel central pathways are involved. New members of the CRH peptide and receptor families have been identified, but roles in the control of reproduction have yet to be determined.

Introduction

Successful passage of genes to the next generation is a key biological drive in all species and the need to reproduce has not diminished with domestication of ruminants. Attainment of optimum fertility under agricultural systems is the key to economic farm production of milk, meat and wool. Conception requires a robust display of oestrous behaviour and ovulation of a mature oocyte coordinated with a supply of competent spermatozoa enabling fertilization in a favourable environment. After luteolysis, the exact timing of events in the follicular phase is dependent on the interplay between GnRH (and thus LH) pulse frequency and amplitude and oestradiol production. This culminates in GnRH and LH surges, stimulation of oestrus behaviour and ovulation (McNeilly *et al.*, 1991).

Email: robsmith@liverpool.ac.uk

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Table 1. Summary of factors influencing GnRH secretion and the possible signalling pathways to the hypothalamus in sheep

Factor	Major signal to the hypothalamus
Stored energy availability	Leptin and insulin
Season (projected nutrient supply)	Melatonin
State of follicular development	Oestradiol
State of possible pregnancy	Progesterone
Unfavourable environment (stress)	CRH, opioids, glucocorticoids

CRH: corticotrophin-releasing hormone.

There have been many definitions of stress but a simple and useful definition is 'any stimulus, real or perceived, which may threaten homeostasis'. Thus, stressors produce signals of (potentially) adverse environmental conditions. The formation of a conceptus marks a commitment to future increased metabolic load. This additional cost may place the mother and the conceptus in jeopardy if environmental conditions become unfavourable. On this basis, animals are subject to evolutionary pressure to ensure that environmental conditions are optimal at the time of maximum metabolic demand (late gestation and lactation). This necessitates the restriction of breeding activity to specific times to ensure future resource availability. In addition, as general environmental conditions cannot be predicted far in advance, if an environment is perceived as at all stressful it would be unsafe to commit to any additional future metabolic load. Thus, suppression of reproduction by stressful stimuli has evolutionary advantages.

Stressors may alter endocrine systems in three distinct ways: (i) as a stimulus-specific response, for example hormones involved in body fluid regulation may be released in response to haemorrhage; (ii) as a generalized response to stimuli (for example, sympathetic nervous system (SNS) activity, hypothalamic–pituitary–adrenal axis (HPA)) or (iii) a hormone may have a key role in normal body function (for example reproduction) and stress may deleteriously alter the hormone signal thus preventing normal function (Smith and Dobson, 2002).

The hypothalamus is the predominant centre for the integration of the many factors that influence control of key body functions, including reproduction. A wide range of physiological signals, acting over diverse time frames, impinge on the hypothalamus to influence the likelihood of a successful reproductive outcome (Table 1). The hypothalamus is the major site of control, and GnRH neurones are pivotal in linking hypothalamic control centres via the hypophysial portal blood system to the rest of the body. However, the pituitary gland and ovaries may also be directly affected.

In experimental studies, both inhibitory and stimulatory effects of a diverse range of stressors modulate various aspects of reproduction. However, even ordinary events in the normal production cycle of domesticated ruminants are stressful and deleterious to reproduction (Dobson and Smith, 2000). As an example of the possible magnitude of stress on fertility, cattle subjected to fixed time AI and subsequent daily blood sampling in an unfamiliar environment had a pregnancy rate of 28% and a large proportion of non-pregnant animals had prolonged follicular phases and cystic ovaries. However, pregnancy rates improved markedly (86%) in animals habituated to the management regimen. This finding indicates that stress-induced delay or failure of ovulation was the prime lesion causing subfertility (Mann, 2001). This review focuses on the mechanisms by which stressors may influence normal hormonal changes in the follicular phase of female ruminants, principally the ewe, to account for such observations.

Impact of study design

Most studies on the effect of stress on reproduction have used animals in the follicular phase, as this is the time of the co-ordinated events that culminate in ovulation and conception. The effect of stress in the luteal phase on reproductive parameters has not been studied extensively. In ewes, cortisol infusion has a greater suppressive effect on subsequent ovulation if it is administered in the follicular rather than the luteal phase (Macfarlane *et al.*, 2000). Moreover, recent studies in primates indicate that stress during the follicular phase can also have detrimental effects on subsequent corpus luteum function (Xiao *et al.*, 2002). This review will concentrate on the greater body of knowledge generated by studies carried out in the follicular phase.

Interpretation of experimental results is dependent on actual study designs. This is very marked for experiments studying the effects of stress as even experimental procedures may interfere and be perceived as stressful. Furthermore, appreciation of stressor-induced endocrine changes also requires an understanding of all factors that impinge on both the HPA and hypothalamic–pituitary–ovarian axis (HPO).

Experimental protocols involving exposure to psychological and environmental stimuli (isolation/restraint, road transport in a truck) or response to administration of compounds that challenge homeostasis (insulin, endotoxin) have been used to investigate the mechanisms involved in stress-induced suppression of GnRH–LH. All of these stimuli cause an initial robust increase in portal blood concentrations of both corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), leading to adrenocorticotrophic hormone (ACTH) secretion from the pituitary gland. ACTH ultimately stimulates cortisol release from the adrenal glands (Engler *et al.*, 1988; Caraty *et al.*, 1990; Karsch *et al.*, 2002; Smith and Dobson, 2002). Subsequently, concentrations of all hormones within the HPA may decrease due to the combined effect of habituation to the stimulus and cortisol negative feedback (Smith and Dobson, 2002). Individual components of the HPA cascade have also been administered to animals to determine which compounds could mediate the effect of stress on reproduction. However, such pharmacological evidence should be interpreted with care as responses to injections or infusions are not reduced over time by habituation or cortisol negative feedback, as are endogenous stress responses. There is also growing evidence that the magnitude of HPA response is modulated by the sex hormone milieu (Tilbrook *et al.*, 2000).

Several experimental approaches have been used to clarify which parts of the normal reproductive endocrine cascade are most sensitive to the effects of stress. The use of ovary-intact animals permits the impact on LH pulse frequency to be monitored while allowing LH to alter oestradiol production endogenously. Such studies allow subsequent changes in oestradiol to exert feedback control on LH pulse frequency and timing of the periovulatory LH surge. Alternatively, ovariectomized (OVX) animals that have had steroid replacement have been used to study the effect of stressors on LH release without the overlying complication of varying the oestradiol feedback signal. However, steroid replacement regimens do not mimic the natural situation perfectly. Variations between individuals in absolute progesterone concentration and the rate of decrease in plasma progesterone after luteolysis also influence LH release. Therefore, many studies have controlled these variables by imposing an artificial luteal phase with a fixed dose of exogenous (intravaginal) progesterone combined with the removal of any endogenous corpora lutea using prostaglandin $F_{2\alpha}$ (Van Cleeff *et al.*, 1998).

As 97% of LH pulses in peripheral blood are coincident with a GnRH pulse in portal blood, LH can be measured to reflect GnRH pulse frequency and the time of GnRH surge onset (Moenter *et al.*, 1990). The amplitude of LH pulses reflects a combination of GnRH pulse amplitude and pituitary gland sensitivity to GnRH. Pituitary gland sensitivity to GnRH

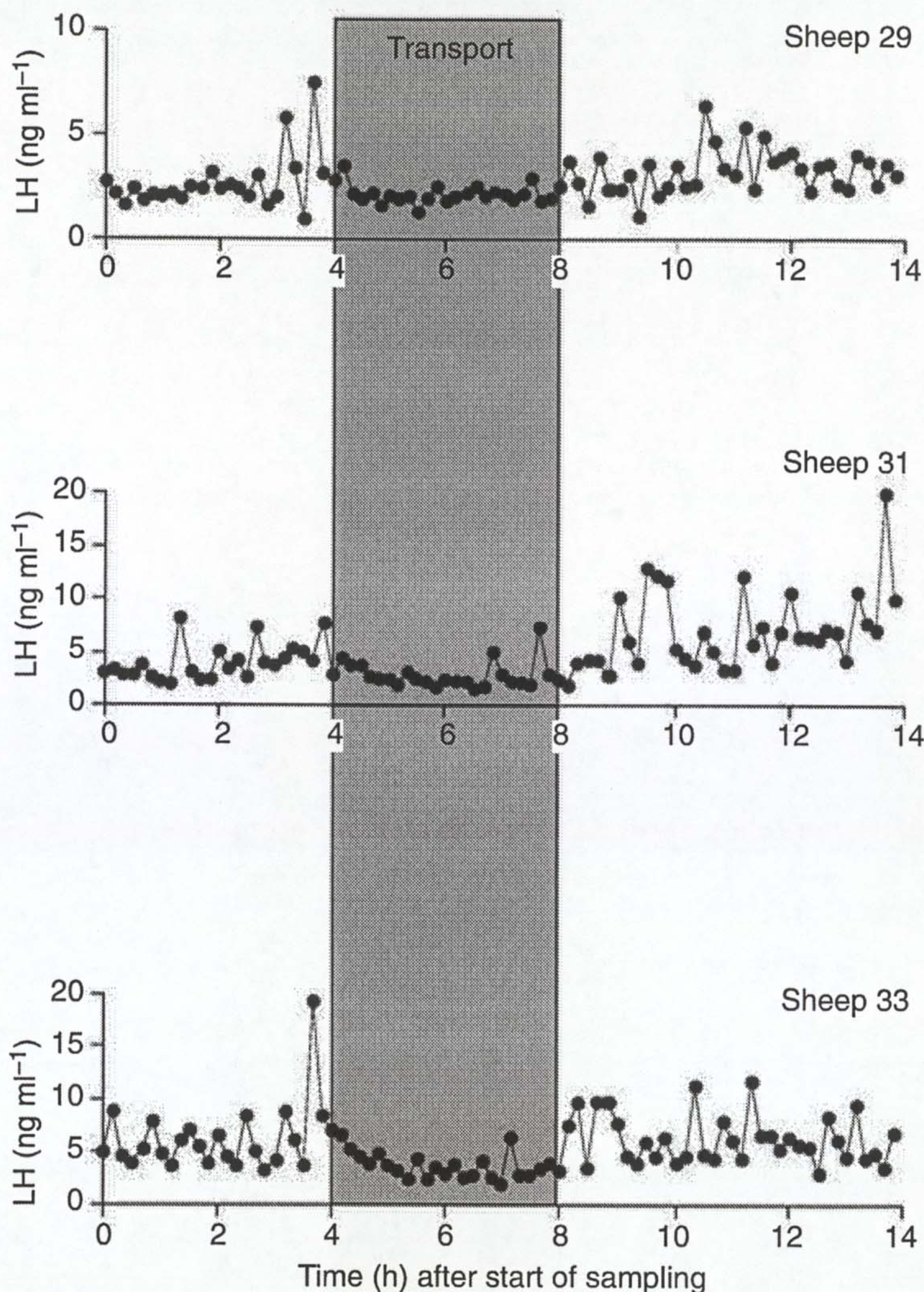

Fig. 1. Profiles of LH in the peripheral plasma of three representative ovariectomized ewes transported in a truck on metalled roads for 4 h (shaded area) in the mid-breeding season without prior steroid treatment. Note different scale on upper profile.

can be studied by challenges with exogenous GnRH but the effect on GnRH self-priming (the ability of one GnRH pulse to increase or maintain the response to the next pulse) requires injection of at least two doses of GnRH (Phogat *et al.*, 1999a). When there is a marked stressor effect at the pituitary gland, LH pulse amplitude may be suppressed to such an extent that measurable LH pulse frequency may be lower than the actual GnRH pulse frequency (Karsch *et al.*, 2002). Thus, the direct measurement of GnRH in hypophysial portal blood remains the gold standard for monitoring the impact of stress on GnRH release. However, LH concentrations may be influenced by loss of GnRH signal during the sampling process itself. This may be the underlying reason why animals prepared for portal blood collection show a longer latency from progesterone removal to LH surge onset than control animals (Dobson *et al.*, 1999).

Effect of stress on GnRH–LH pulsatility

Frequency

GnRH–LH pulse frequency is totally suppressed in some individuals by exposure to stress (Figs 1–3). However, differences between studies in parameters such as season of year and sex hormone treatments affect responses and make direct comparisons difficult (Table 2). The severity of suppression of GnRH–LH pulsatility appears to parallel the degree of HPA stimulation both within and between studies. The rank order of stressor severity in commonly applied models increases through isolation, transport, insulin-hypoglycaemia to the most severe, endotoxin administration (Dobson and Smith, 1998; Tilbrook *et al.*, 2000; Karsch *et al.*, 2002). Daily repetition of stress leads to habituation of the inhibitory effect on LH pulse frequency and amplitude in a similar manner to that during habituation of the HPA response (Rasmussen and Malven, 1983). However, prolongation of stress does not always lead to continued suppression of LH pulse frequency. For example, LH pulse frequency is only significantly reduced for the first 4 h of an 8 h transport period (Phogat *et al.*, 1999a), and during prolonged hypoglycaemia LH pulsatility resumes, whereas blood glucose concentrations are still suppressed (H. Dobson and R. F. Smith, unpublished).

Amplitude

LH pulse amplitude is decreased by almost all the stressors listed above and a reduction in GnRH pulse amplitude by endotoxin has been observed (Karsch *et al.*, 2002). However, gonadotroph responsiveness to GnRH can also be reduced by the severe stress of endotoxin (Karsch *et al.*, 2002); and although moderate transport stress does not suppress the response to one injection of exogenous GnRH, it does reduce GnRH self-priming which results in lower LH responses to subsequent GnRH pulses (Phogat *et al.*, 1999a).

Consequences at the ovary

In ovary-intact animals, stress-induced lowered LH pulsatility slows follicular growth and reduces ovarian oestradiol production. This finding has been confirmed by administering exogenous LH pulses to mimic those observed during transport in ewes bearing ovarian autotransplants and treated with GnRH antagonists to suppress endogenous LH production (Dobson and Smith, 1998). A reduction in oestradiol production in ovary-intact animals may be the cause of the increased delay in the onset of the LH surge observed in these animals compared with OVX and oestradiol-treated animals, when all are transported just before the time of the expected LH surge (Dobson and Smith, 1998).

Effects of stress on the GnRH–LH surge

Timing of onset

Three phases in the induction of an oestradiol-induced GnRH–LH surge have been identified: (i) activation, (ii) transmission and (iii) surge secretion (Fig. 4) (Evans *et al.*, 1997). Transport blocks or delays the LH surge of intact ewes when it is timed to coincide with the late transmission or early surge secretion phases (Dobson and Smith, 2000). However, hypoglycaemia (10–12 h) induced during any of the phases delays the surge in oestradiol-treated OVX ewes (Medina *et al.*, 1998). Moreover, endotoxin administration blocks activation and the early transmission phase but was without affect when applied during the late transmission

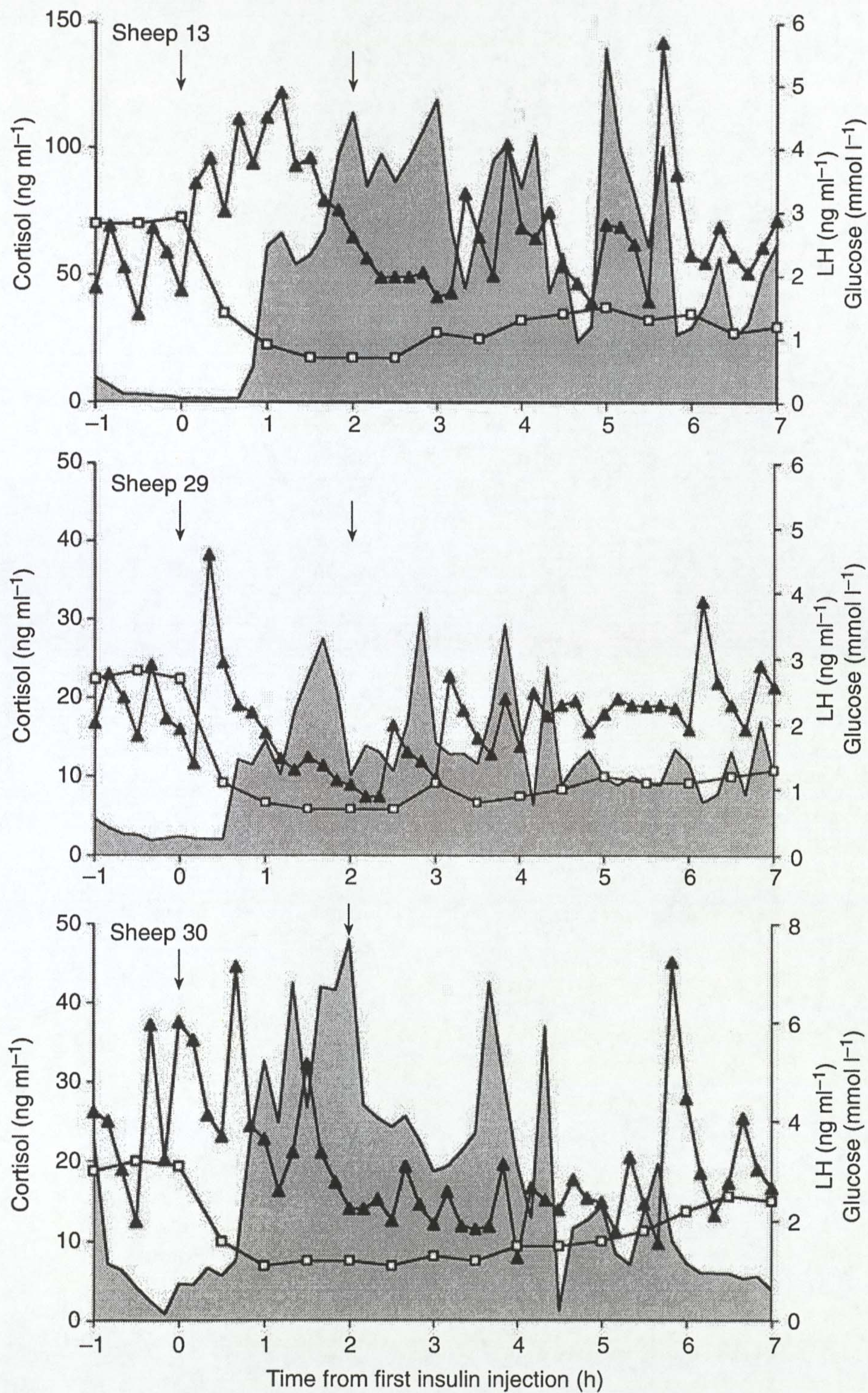

Fig. 2. Profiles of cortisol (shaded area), LH (▲) and glucose (□) in peripheral plasma of three representative ovariectomized ewes administered 1 iu insulin kg^{-1} at 0 and 2 h (arrows) in the mid-breeding season without prior steroid treatment. Note different scales on each profile.

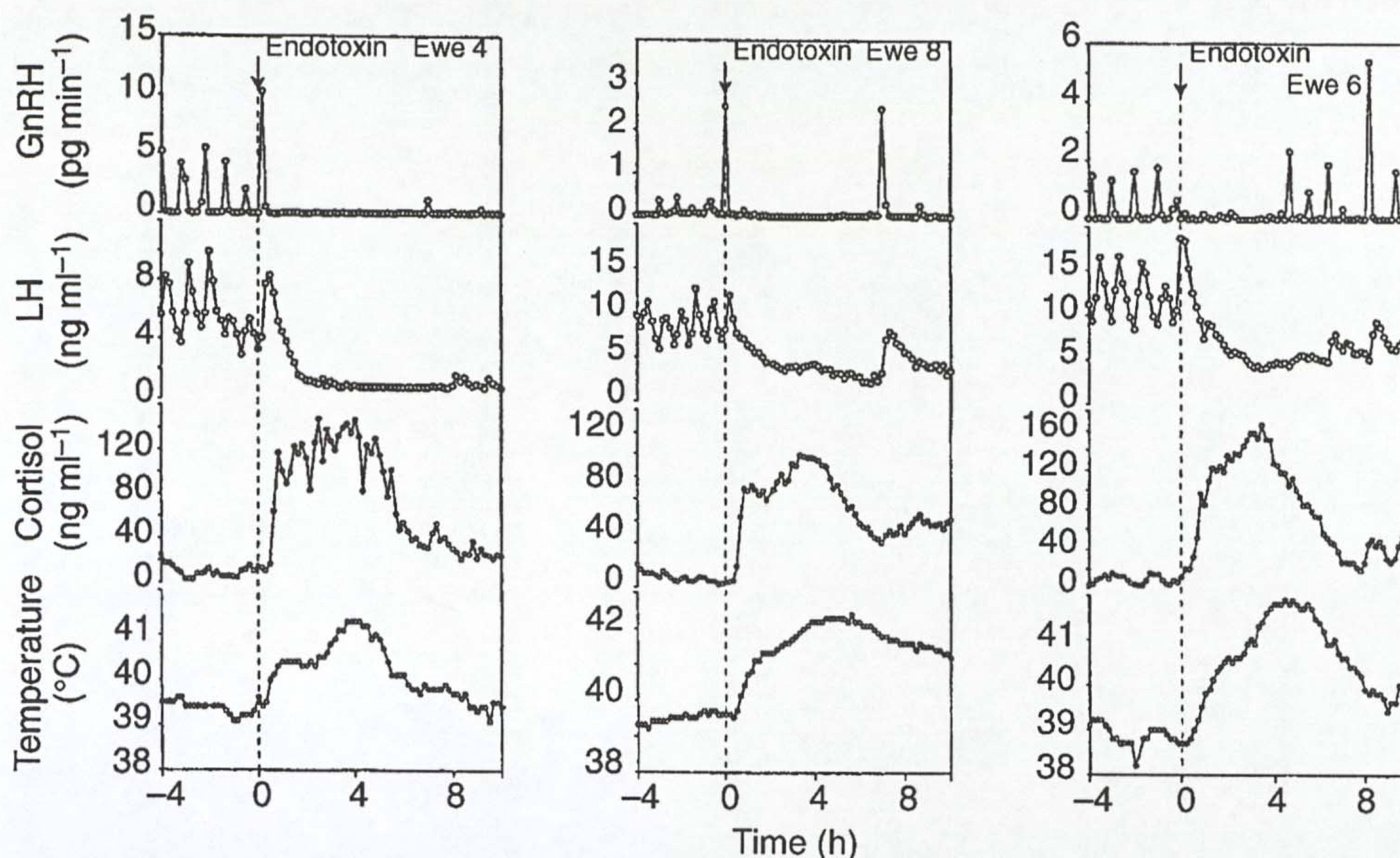

Fig. 3. Profiles of GnRH in portal plasma, LH and cortisol in the peripheral plasma and core body temperature of three representative ovariectomized ewes that were not treated with steroid but received endotoxin (400 ng kg^{-1}) at 0 h (dashed vertical line). Note different scales on each profile. (Reproduced from Battaglia DF, Bowen JM, Krasa HB, Thrun LA, Viguie C and Karsch FJ (1997) Endotoxin inhibits the reproductive neuroendocrine axis while stimulating adrenal steroids: a simultaneous view from hypophyseal portal and peripheral blood *Endocrinology* **138** 4273–4281. Copyright owner, The Endocrine Society).

or surge secretion phases (Karsch *et al.*, 2002). Isolation or restraint can also block the surge when applied in the late transmission phase, but effects during other phases have not yet been investigated (H. Dobson and R. F. Smith, unpublished). Identification of the mechanisms underlying the differential effects of various stressors could give important insights into the locations and neurotransmitters involved in the three phases during normal GnRH–LH surges.

Amplitude and duration

There are also differential effects of stressors on surge amplitude depending on the phase of the surge mechanism during which the stress is applied (Fig. 4), but there are no stress-induced changes in the duration of the surge. Transport during the late transmission phase suppresses surge amplitude in both ovary intact and OVX oestradiol-treated ewes (Dobson and Smith, 1998). Hypoglycaemia also reduces surge amplitude when applied during the transmission phase in oestradiol-treated OVX ewes. Conversely, hypoglycaemia or endotoxin administration during the activation phase had no effect on the amplitude of the surge. Endotoxin administration during the transmission phase was also without effect on either LH surge parameter (Karsch *et al.*, 2002). The differential effects of different stressors on each phase of the GnRH–LH surge provide an opportunity to identify which neuronal pathways are active in each phase by studying differential neuronal activation during each stress.

Table 2. Summary of LH pulse frequency and amplitude data from different studies with stimuli of increasing severity in the ewe during the breeding season

Stimulus	Gonadal status	Steroid status	Pulse frequency h^{-1}		Pulse amplitude (ng ml^{-1})		Reference
			Before stress	During stress	Before stress	During stress	
Isolation / restraint 2 h	OVX	None	1.1 ± 0.1	0.44 ± 0.1*	5.2 ± 1.0	2.8 ± 0.5*	Rasmussen and Malven, 1983
Isolation / restraint 4 h	OVX	None	1.9 ± 0.3	1.0 ± 0.2*	1.9 ± 0.9	1.3 ± 0.2	Tilbrook *et al.*, 2000
		2 cm × 1 cm oestrogen implant	2.5 ± 0.3	1.6 ± 0.2*	0.9 ± 0.1	0.4 ± 0.1*	
		CIDR progesterone	0.7 ± 0.1	0.3 ± 0.1*	5.2 ± 1.9	3.1 ± 0.8	
		2 cm × 1 cm oestrogen implant and CIDR progesterone	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1*	
Transport for first 4 h of 8 h	Intact	Follicular phase	0.7 ± 0.1 (controls)	0.2 ± 0.1*	–	–	Phogat *et al.*, 1999a
Transport 4 h	Intact	Follicular phase	1.0 ± 0.2	0.8 ± 0.3	0.9 ± 0.3	0.3 ± 0.1*	Dobson *et al.*, 2000
Transport 4 h	OVX	None	1.8 ± 0.1	1.6 ± 0.1*	2.7 ± 0.5	1.7 ± 0.2*	Dobson *et al.*, 1999
Transport 4 h	OVX	1 cm oestrogen implant	1.8 ± 0.1	1.6 ± 0.1*	2.7 ± 0.4	1.7 ± 0.2*	Dobson *et al.*, 2000
Transport 4 h	OVX	2 cm × 3 cm oestrogen implant	0.5 ± 0.2	0.8 ± 0.3	0.2 ± 0.1	0.1 ± 0.1	Dobson *et al.*, 1999
Hypoglycaemia (1 iu insulin kg^{-1})	OVX	3 cm oestrogen implant	1.7 ± 0.1	1.5 ± 0.1	2.2 ± 0.4	1.9 ± 04	Dobson *et al.*, 2000
Hypoglycaemia (2 iu insulin kg^{-1})	OVX	None	1.3	0.4*	3.2 ± 1.1	1.4 ± 0.5	Clarke *et al.*, 1990
Hypoglyaemia (5 iu insulin kg^{-1})	OVX	None	1.3 ± 0.1	0.6 ± 0.1*	–	–	Medina *et al.*, 1998
Endotoxin (400 ng kg^{-1})	OVX	None	1.1 ± 0.1	0.3 ± 0.1*	13.0 ± 2.0	6.0 ± 2.0	Battaglia *et al.*, 1997

Within a row (experiment) an * indicates significant reduction in parameter during stress compared with before stress ($P < 0.05$).
CIDR: controlled internal drug releasing device; OVX: ovariectomized.

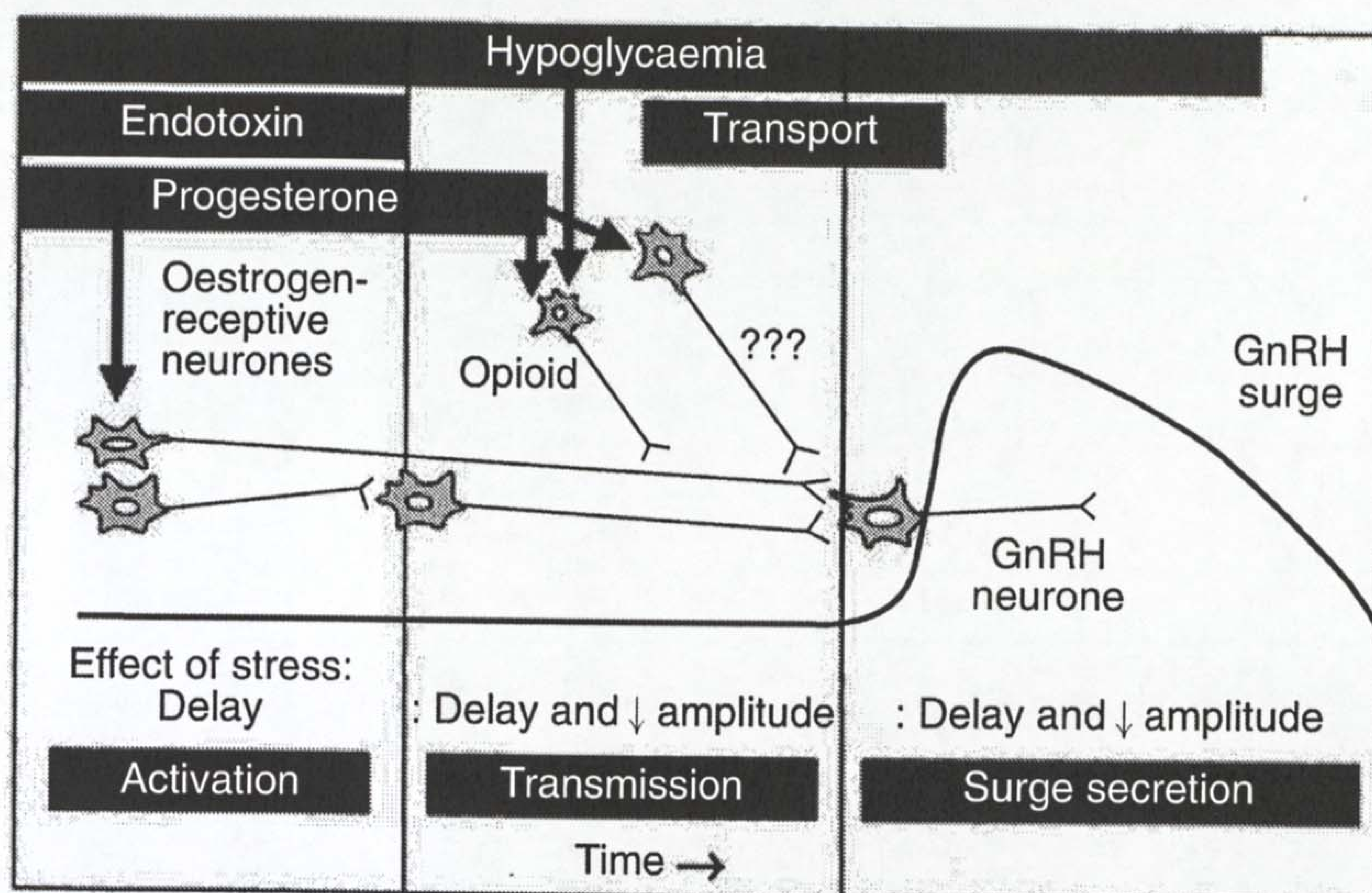

Fig. 4. Representation of the phases during induction of a GnRH surge when transport, insulin-induced hypoglycaemia, endotoxin or progesterone can delay the surge with possible neurocircuitry also shown. Adapted from Evans *et al.* (1997). Data from Smart (1994), Medina *et al.* (1998) and Karsch *et al.* (2002).

Possible HPA mediators of the effect of stress on reproduction

In the following summary, data from sheep will be discussed; when not available or contradictory, reference will be made to studies in other species for clarification. An in-depth review of potential intra-hypothalamic pathways mediating the effect of stress on reproduction has been published (Dobson *et al.*, 2003).

Arginine vasopressin

Arginine vasopressin (AVP) is the most abundant hypothalamic hormone secreted from the paraventricular nucleus (PVN) of the hypothalamus in response to stress and is the main stimulus for ACTH secretion in sheep (Smith and Dobson, 2002). However, intracerebroventricular injection of AVP alone in males or in combination with CRH in OVX females fails to alter LH secretion in sheep, although AVP suppresses LH release in primates (Heisler *et al.*, 1994; Tilbrook *et al.*, 2000). The involvement of AVP receptors in stress-induced inhibition of GnRH–LH has not been studied in ruminants to date. Studies in female primates have been contradictory: hypoglycaemia-induced suppression of LH release has been both inhibited and permitted by AVP receptor antagonism (Heisler *et al.*, 1994; Chen *et al.*, 1996). The role of AVP is no clearer in rats. High doses of AVP suppress LH, but AVP antagonism does not reverse hypoglycaemia-induced suppression of LH release (Cates *et al.*, 1999). Moreover, pretreatment with AVP-antiserum prolongs the inhibitory effect of CRH on the LH surge (Roozendaal *et al.*, 1996). Further studies in ruminants are required but it should be noted that no cells express AVP mRNA in the bed nucleus of the stria terminalis (BNST), the amygdala, or the medulla and brainstem regions (Matthews *et al.*, 1993). It has been suggested that these are key control areas for stress-induced suppression of LH in rats (Rivier and Rivest, 1991).

Corticotrophin releasing hormone

Corticotrophin releasing hormone (CRH) is the predominant corticotrophin-releasing compound in rats and has an important role facilitating AVP action in sheep. In most species, both central and peripheral administration of CRH suppresses LH secretion in the majority of studies. Oestrus and ovulation do not occur in intact ewes infused with CRH i.c.v. (Polkowska and Przekop, 1997). Furthermore, GnRH self-priming is reduced by CRH *in vitro* (Smart, 1994). However, restraint stress can still suppress LH secretion in CRH-deficient mice (Jeong *et al.*, 1999). The results of the last study either questions the requirement for CRH or indicates the existence of compensatory mechanisms in neuroendocrine systems.

The situation in sheep is yet more perplexing. In three laboratories, i.c.v. administration of CRH increased LH pulse frequency and mean LH concentration. All of these studies used gonadectomized animals and responses were obtained only in steroid pre-treated animals of either sex (Naylor *et al.*, 1990; Caraty *et al.*, 1997; Tilbrook *et al.*, 2000). Endogenous stimulation of LH pulsatility also occurred when OVX ewes were transported 38 h after removal of exogenous oestradiol and progesterone implants during the transition into anoestrus (Fig. 5). There was no difference in the cortisol response to stress between animals. However, an increase in LH pulsatility was observed during stress in ewes that had entered seasonal oestradiol negative feedback and had few or no LH pulses during the control period. The ewes that still had LH pulses were suppressed as usually observed in the breeding season (Dobson *et al.*, 1999), thus steroid negative feedback may be required for CRH stimulation of LH to occur. It is proposed that either exogenous CRH or stress-induced endogenous CRH overcomes the inhibitory effect of steroids on LH rather than CRH directly stimulating LH pulsatility.

CRH has been proposed as a major intra-hypothalamic mediator of the effects of stress (Rivier and Rivest, 1991), but how does it reach its site(s) of influence? There are insufficient data to propose a model based solely on work in ewes. The following observations can be made based on knowledge of neurocircuitry present in sheep and functional data from rats. Major CRH neurones pass from the PVN to the external layer of the median eminence (ME). These terminals are not closely associated with the GnRH-rich areas indicating that the ME does not have major importance in CRH–GnRH interactions. CRH fibres also project rostrally from the PVN towards the mPOA, but have not been traced further forward than the anterior commissure (Paull *et al.*, 1982). Secretory activity of most CRH neurones in the PVN is suppressed by basal and stress-induced cortisol concentrations, but activation of these CRH neurones during removal of corticoid suppression does not inhibit GnRH–LH in sheep or primates (Van Vugt *et al.*, 1997; Debus *et al.*, 2002). Moreover, lesions to the PVN do not prevent footshock-induced suppression of LH in rats (Rivier and Rivest, 1991). These observations indicate that the PVN neurones projecting to the ME are not involved in stress-induced suppression of GnRH–LH.

Important CRH-positive synapses have been identified on GnRH perikarya in the mPOA in rats, but not yet in ewes. CRH is present in the ovine mPOA. Although CRH mRNA has not been identified there, neurones do project to the mPOA from several other brain regions expressing CRH mRNA (Palkovits *et al.*, 1983; Matthews *et al.*, 1991). For example, the CRH content of the amygdala increases in response to a barking dog, and fibres from the amygdala innervate the mPOA in ewes, although the identity of these fibres has not yet been determined (Palkovits *et al.*, 1983; Cook, 2001). In addition, CRH cells are present in the hindbrain (locus coeruleus and nucleus of the solitary tract) in ewes and noradrenergic neurones project from these nuclei to the mPOA (Palkovits *et al.*, 1983; Matthews *et al.*, 1991). Lesions of the locus coeruleus in rats suppress GnRH release (Anselmo Franci *et al.*, 1997).

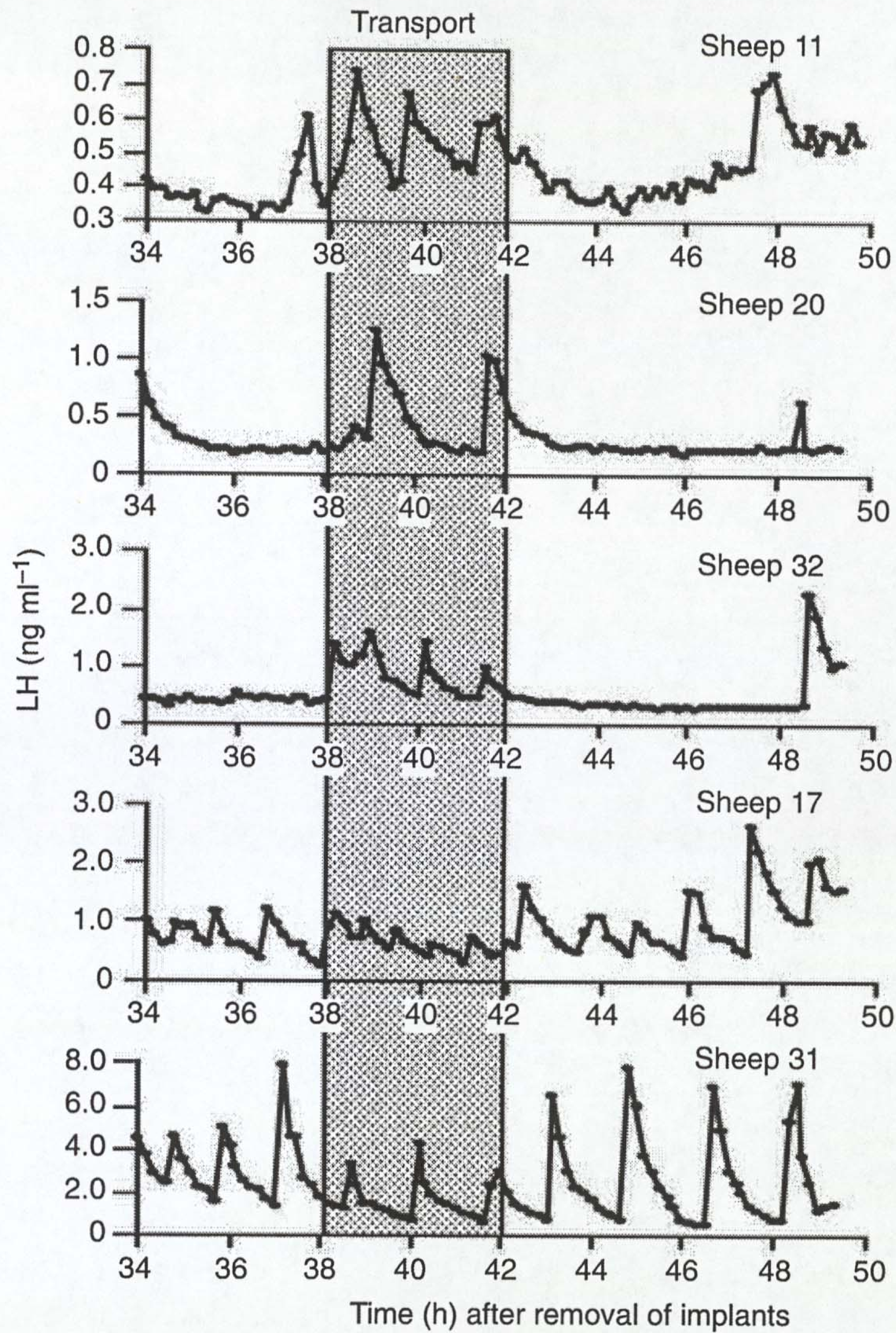

Fig. 5. Profiles of LH in the peripheral plasma of five representative ovariectomized ewes that were transported after steroid removal during transition into anoestrus (shaded area) for 4 h. Note stimulation of LH during transport in first three ewes (sheep, 11, 20 and 32) that were suppressed before transport and suppression of pulse amplitude in the last two ewes (sheep 17 and 31) that were actively pulsing before transport. Data obtained from Dobson *et al.* (1999) with permission.

However, CRH injection into the locus coeruleus increases noradrenaline release and, thus, may stimulate rather than suppress GnRH release (Schulz and Lehnert, 1996). The arcuate nucleus also contains CRH in ewes (Palkovits *et al.*, 1983). Antagonism of CRH within the rat arcuate nucleus–ME results in a decrease in beta-endorphin and an increase in GnRH release (Nikolarakis *et al.*, 1988). Thus, there are several locations at which CRH is present in ewes and could influence GnRH release.

Other CRH-like peptides and CRH receptors (CRH-R) may also be involved in the control of GnRH. The family includes three ligands: urocortin, urocortin II (stresscopin-related peptide) and urocortin III (stresscopin) and two receptors. CRH has higher affinity for CRH-R1 than CRH-R2. Conversely, urocortin preferentially binds to CRH-R2 > CRH-R1, whereas urocortin II and III act only on CRH-R2. Both CRH-R1 and CRH-R2 are expressed in GnRH cell lines (Tellam *et al.*, 2000). Urocortin has more limited distribution in sheep than in rat brain and is predominantly localized in the Edinger–Westphal nucleus, and several cranial nerve nuclei (Cepoi *et al.*, 1999). Urocortin stimulates LH pulsatility when administered i.c.v. in OVX ewes. However, LH was stimulated only on day 3, and not on day 1 or day 5, of a 5 day experiment using linearly increasing doses of urocortin (Holmberg *et al.*, 2001). The distribution of urocortin II or III in ruminants has not been studied to date. In rats, urocortin III neurones are in the median preoptic nucleus and in the rostral perifornical area lateral to the PVN. Urocortin III fibres innervate the ventromedial nucleus, medial preoptic nucleus, and ventral premammillary nucleus, the lateral septum, the BNST, and the medial nucleus of the amygdala. Most of these areas also express high concentrations of CRH-R2 (Li *et al.*, 2002). The possibility that these new CRH-related peptides and receptors are involved in the control of reproduction warrants further study and may clarify the contradictions between species reported so far for CRH.

Adrenocorticotrophic hormone

Many studies have used adrenocorticotrophic hormone (ACTH) to stimulate adrenal cortisol (and progesterone) secretion. Most of these studies use high doses of ACTH for several days which circumvents hypothalamic feedback mechanisms and, thus, are not representative of normal HPA function. Treatment of cattle in the follicular phase with ACTH for several days suppresses LH pulse frequency, decreases oestradiol production, increases progesterone and cortisol concentrations and, thus, delays onset of the GnRH–LH surges and behavioural oestrus. A high proportion of animals treated in this way develop follicular cysts (Dobson *et al.*, 2000). Single injections of ACTH during the transmission phase of the GnRH–LH surge induction mechanism delay or prevent the LH surge. Interestingly, animals that did not have an LH surge tended to have higher plasma progesterone concentrations than those that did have an LH surge (Phogat *et al.*, 1999a). Both *in vivo* and *in vitro* studies also indicate that ACTH has a direct action within the pituitary gland to reduce GnRH self-priming (Phogat *et al.*, 1997, 1999b). It is likely that the effect of ACTH on LH surge secretion is mediated by progesterone, but the direct action of ACTH on the gonadotroph may be significant during stress.

Cortisol

In a similar manner to ACTH, cortisol has been administered to produce plasma concentrations of cortisol similar to the maximum observed during stress. For example, cortisol administered to obtain a plasma concentration of 70 ng ml^{-1} for 5 days beginning during the follicular phase suppresses follicular development and blocks the LH surge and ovulation (Macfarlane *et al.*, 2000). This concentration is similar to peak concentrations seen in response to stress (Figs 2 and 3). However, even with a constant stimulus, cortisol concentrations decrease below peak values within a few hours due to habituation and cortisol negative feedback, thus prolonged constant infusion studies do not mimic physiological events (Smith and Dobson, 2002). *In vitro* cortisol suppresses both basal and GnRH-stimulated LH release from cattle pituitary glands (Li and Wagner, 1983). Debus *et al.* (2002) reported that shorter periods of cortisol infusion to mimic that seen in response to endotoxin suppresses LH

pulsatility acutely. However, blockade of cortisol synthesis did not prevent inhibition of LH pulsatility due to endotoxin administration, leading to the conclusion that increased cortisol itself was sufficient to account for suppression of LH pulsatility seen but the cortisol rise was not necessary for this suppression. The medial preoptic area and arcuate nucleus contain high concentrations of type II glucocorticoid receptors which are suppressed by oestradiol treatment. Like many other neurotransmitters and receptors that are involved in the control of GnRH, the glucocorticoid receptors are not co-localized with GnRH (Dufourny and Skinner, 2002). Thus, cortisol may be a significant mediator of the suppression of fertility due to stress but determining whether it is obligatory for any stimulus requires further research.

Progesterone and opioids

Plasma concentrations of progesterone, presumably secreted by the adrenal glands, increase during stress. Progesterone concentrations increase significantly from 0.11 to 0.78 ng ml^{-1} during endotoxin administration and from 0.30 to 0.38 ng ml^{-1} during transport (Phogat *et al.*, 1999a; Karsch *et al.*, 2002). Administration of near-luteal phase concentrations of progesterone (7 ng ml^{-1}) blocks both LH pulsatility and the GnRH–LH surge if administered during the activation and early transmission phases, but not later. The effects of progesterone on pulsatility and during the early transmission phase are mediated by endogenous opioids (Richter *et al.*, 2001), and arcuate nucleus opioid neurones projecting to the mPOA or ME may be the source (Conover *et al.*, 1993). Opioids are also involved in suppression of LH pulsatility and the delay in LH surge during hypoglycaemia (Clarke *et al.*, 1990; Dobson and Smith, 2000). However, this delay is not prevented by antagonism of nuclear progesterone receptors using RU486 (Dobson and Smith, 2000).

Thus, the inhibitory effects of stress or progesterone during pulsatile LH secretion and the transmission phase of the LH surge could be mediated via a common opioid interneurone, or a separate system. However, overall, these findings indicate that the low concentrations of progesterone secreted in response to stress are not involved in a genomic inhibition of the LH surge. This does not preclude a role for the higher concentrations of progesterone induced by prolonged doses of ACTH used in some studies. For example, low doses of progesterone are as effective as ACTH at experimentally producing follicular cysts (Dobson *et al.*, 2000).

Additional pathways identified for specific stressors

Endotoxin

In vivo, low doses of endotoxin act on the pituitary gland, whereas high doses act via both the hypothalamus and pituitary gland to suppress both pulsatile and surge LH secretion (Karsch *et al.*, 2002). There is evidence that this effect is mediated via prostaglandin synthesis (in ewes) and opioids (in heifers) (Kujjo *et al.*, 1995; Karsch *et al.*, 2002). Studies in rats indicate that endotoxin also acts via interleukin-1α and -β synthesized within the brain, and GABA release in the hypothalamus (Feleder *et al.*, 1996). This is in addition to the increase in c-*fos* expression induced by endotoxin in many of the sites already implicated in stress-induced suppression of LH pulsatility (OVLT/medial preoptic area, PVN, arcuate nucleus/ME, central nucleus of the amygdala, locus coeruleus, nucleus of the solitary tract, area postrema and ventrolateral medulla) (Nappi *et al.*, 1997).

Hypoglycaemia

Opioids mediate LH suppression both in sheep during insulin-induced hypoglycaemia and in rats after administration of the glucose antagonist, 2-deoxyglucose (2DG) (Briski and Sylvester, 1999; Dobson and Smith, 2000). Nitric oxide may also be involved as 2DG elicites c-*fos* expression in neurones that produce nitric oxide in the mPOA, PVN and BNST in rats (Briski and Sylvester, 1999). The area postrema contains glucose-sensitive neurones that detect (relative) hypoglycaemia in rats and sheep, and ablation of this area in rats prevents the inhibition of LH pulsatility induced by insulin (Cates and O'Byrne, 2000; Ohkura *et al.*, 2000). However, Adam and Findlay (1998) were unable to detect c-*fos* expression in the area postrema during insulin treatment in sheep. Conversely, another study on sheep identified c-*fos* in the area postrema after insulin-induced hypoglycaemia, as well as isolation and transport simulation (Vellucci and Parrott, 1994). Endotoxin administration also stimulates c-*fos* expression in the area postrema in rats (Nappi *et al.*, 1997) indicating that this area may be important in the response to all stressors, not just to hypoglycaemia.

Conclusions

Limits on reproductive efficiency due to management and environmental factors imposed on domesticated ruminants have a major impact on economics of farm production. Reproductive efficiency is clearly an objective indicator of animal welfare. The relative importance of interference at each level of the reproductive system needs clarification along with all of the interrelationships involved. The role of all neurones that impinge upon the key control point, the GnRH neurone, requires identification. Elucidation of the mechanisms involved in stress-induced suppression of reproduction may allow the development of strategies to ameliorate the deleterious effects. The significance of the ever increasing range of candidate neurotransmitters requires further study in ruminants.

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