The role of the melanocortin system in the control of reproduction in the ewe

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This thesis is presented for the degree of Doctor of Philosophy at

Monash University

For my wonderful Grandma who I know would be proud.

ERRATA

P 30, 1.9.6 Heading; “Agouti regulated peptide” should read “Agouti related protein”

P 43, Para 3: “Y2 antagonist” should read “Y2 agonist”

P 31, Fig 1.5; Add at the end of figure legend: “VFI = Variable food intake, μ and δ are central opioid receptors

P 40, line 3: “ORX-1” should read “ORX-1R”

P 94, line 12: “changes” should read “changed”

P 109, Fig 4.1: Add at the end of legend: “n=5ewes/group”

P 118, Fig 4.6, line 8: “kisspeptin cells provide synaptic…” should read “POMC cells provide synaptic…”

Page 121, line 13 and 14: References “7,34” should read “Smith *et al,* 2006”

P 127, last line: “associated hypothalamic amenorrhea” should read “associated with hypothalamic amenorrhea”

P135, 3rd reference: Add “150(7):3214-20

P153, 10th reference: Add “90(1):31-53

P153, 11th reference: Add “21(8):690-7

P153 last reference: Add “150(6):2805-12

P158,10th reference: Add “doi:10.1016/j.numecd.2009.04.007”

ADDENDUM

P 13, Para 3: Add at end of last para: In addition to affecting food intake, estradiol also affects sex behaviour, but it is not known whether it works via the same or different brain areas. Dilute estradiol implants in the VMH that are sufficient to elicit lordosis fail to decrease food intake, whereas the same diluted implant into the PVN decreases food intake but does not permit lordosis (Butera and Beikirch, 1989). The PVN is therefore necessary for the effects of estradiol on food intake but not for the effects of estradiol on lordosis, suggesting neuroanatomical separation.

Add to reference list:

Butera PC, Beikirch RJ (1989) Central implants of diluted estradiol: independent effects on ingestive and reproductive behaviors of ovariectomized rats. Brain Res 491:266-273.

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Summary

Altered metabolic status and steroid milieu are relayed to the brain to regulate the secretion of gonadotropin releasing hormone (GnRH), the gatekeeper of the neuroendocrine reproductive axis, but the means by which signals from the periphery are relayed to GnRH cells is unclear. The melanocortins, encoded by the proopiomelanocortin (POMC) gene, are poised to act as a conduit for metabolic and sex steroid feedback to GnRH cells. Although data from the rodent species indicate a role for the melanocortins as a positive regulator of the reproductive axis, little work has been carried out in the ovine species. Furthermore, the neuronal circuitry by which the melanocortins exert their effect on the reproductive axis is yet to be delineated.

The first study (Chapter 2) aimed to test the hypothesis that adipose hormone, leptin, is capable of restoring gonadotropic function by up regulating central acetylated melanocortin production in the ewe. The results showed that intracerebroventricular (icv) infusion of leptin was able to correct the hypogonadotropic state in the lean condition with a concomitant up regulation of POMC. Furthermore, administration of a melanocortin agonist, MTII, into the brain by icv infusion activated pulsatile LH secretion (reflecting GnRH secretion) in our lean ewe model. A particularly salient finding of Chapter 2 was the decrease in acetylated α-MSH in the terminal beds of lean hypogonadotropic ewes, and it is proposed that leptin treatment increases the transport and acetylation of melanocortins to the synaptic boutons of POMC neurons in the brain.

In Chapter 3, the objective was to further substantiate the stimulatory role of the melanocortins on the reproductive axis in the ewe, and the associated aim was to delineate how 'dialogue' is achieved between melanocortins and GnRH cells. It was revealed that central treatment with a melanocortin agonist was able to overcome the negative feedback effects of sex steroids in the luteal phase of the estrous cycle, to stimulate pulsatile LH secretion. The same treatment was unable to induce an LH surge or ovulation in seasonally anestrous ewes, but a transient rise in basal LH secretion was evident. Few melanocortin producing cells of the

arcuate nucleus (ARC) were found to project to the preoptic area (POA) where GnRH neurons are located, and an interneuronal pathway is proposed, consistent with previous work from this laboratory. Orexin (ORX) mRNA in the dorsomedial hypothalamus (DMH) and kisspeptin (Kiss1) mRNA in the POA were up regulated following melanocortin treatment in the luteal phase, illustrating potential novel conduits for melanocortin regulation of GnRH cells.

The studies conducted in Chapter 4 were undertaken to decipher the neuronal circuitry through which the melanocortins might act to stimulate the reproductive system. The results confirmed and extended data obtained in rodent species that illustrate leptin regulation of the kisspeptin system. Using real-time polymerase chain reaction (RT-PCR) of single cells, the presence of the long form leptin receptor (Ob-Rb) was demonstrated in kisspeptin neurons of the ARC and POA. This allows for facilitation of the actions of melanocortins to regulate metabolic and reproductive homeostasis, via kisspeptin cells. Novel reciprocal pathways between the kisspeptin and the POMC/NPY systems were shown using Zeiss Apotome technology. The mutual communication between these systems may aid in the transmission of sex steroids and leptin to regulate reproductive and metabolic function within the brain, and a model is proposed for this.

Collectively, the body of work presented in this thesis presents evidence that the melanocortins act within the brain to positively regulate the neuroendocrine reproductive axis in the ewe. Thus, melanocortin cells relay information regarding leptin status and sex steroid feedback to the brain via an interneuronal pathway to regulate both reproductive and metabolic homeostasis. Understanding the involvement of the melanocortin system in the stimulation of the reproductive axis during times of reproductive suppression, will aid in the therapeutic intervention of hypothalamic amenorrhea.

Declaration

I hereby declare that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis, and that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university. I also declare this thesis is less than 100,000 words in length exclusive of tables, references and appendices.

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This thesis includes 3 original papers which are either published, submitted or in preparation to be published in peer reviewed journals. The core theme of the thesis is the role of the melanocortin system in the control of reproduction in the ewe. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Physiology under the supervision of Prof. Iain Clarke (Monash University).

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2-4 my contribution to the work involved the following:

Thesis chapter	*Publication title*	*Publication status**	*Nature and extent of candidate's contribution*
2	Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes	Accepted	Experimental conduct, Laboratory analysis, Data collection, Statistical analysis, Preparation of manuscript
3	Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial nucleus and kisspeptin neurons in the preoptic area of the ewe	Provisionally accepted	Experimental conduct, Laboratory analysis, Data collection, Statistical analysis, Preparation of manuscript
4	Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells to stimulate reproduction	In preparation for submission	Experimental conduct, Laboratory analysis, Data collection, Statistical analysis, Preparation of manuscript

I have renumbered and rearranged sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed:……

Kathryn Backholer
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Acknowledgements

Wow I can't believe it's all over!!! The completion of a PhD thesis is not just the culmination of only one persons work, but is the result of assistance and support from many other people. I therefore wish to thank numerous people whose contribution has enabled the completion of this journey. First and foremost I wish to thank my supervisor Professor Iain Clarke. Thanks Iain for all your support and guidance over the past three and a half years and for your unwavering optimism which kept me motivated when nothing seemed to work. You have taught me a great deal and I truly appreciate everything you have done for me.

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Finally, to my Pete, thanks for always listening and for always dreaming along with me.

List of Publications

Publications arising from thesis:

Backholer K., Bowden M., Gamber K., Bjørbæk C., Iqbal J., Clarke IJ. (2009) Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes. Accepted into *Neuroendocrinology*

Backholer K., Smith JT., Clarke IJ. (2009) Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial nucleus and kisspeptin neurons in the preoptic area of the ewe. Provisionally accepted into *Endocrinology*

Backholer K., Smith JT., Iqbal J, Clarke IJ. (2009) Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells to stimulate reproduction. In preparation to be submitted to *Journal of Neuroscience*

Abstracts / Conference Proceedings:

Backholer K., Smith JT., Clarke IJ. Kisspeptin cells project to proopiomelanocortin and neuropeptide Y cells in the arcuate nucleus of the ewe; evidence for transmission of sex steroid feedback to appetite regulating cells. Endocrine Society of Australia (ESA). Christchurch, New Zealand (2007)

Backholer K., Bowden M., Gamber K., Bjorbaek C. Clarke IJ. Proopiomelanocortin, melanocortin, and reproductive function in lean body condition. The Endocrine Society's Annual Meeting. San Francisco, USA (2008)

Backholer K., Clarke IJ. Melanocortin stimulation of neuroendocrine reproductive function. Endocrine Society of Australia (ESA). Melbourne, Australia (2008)

Abbreviations and Symbols

The abbreviations listed below apply to the entire text, excluding legends of tables and figures where names of structures and abbreviations appear separately. Abbreviations are written in full on first appearance in each chapter.

Abbreviations

AC	anterior commissure
aCSF	artificial cerebral spinal fluid
ACTH	adrenocorticotropic Hormone
Act-α-MSH	acetylated alpha melanocyte stimulating hormone
Ad lib	ad libitum
AGRP	agouti related protein
ANOVA	analysis of variance
ARC	arcuate Nucleus
AVPV	anteroventral periventricular nucleus
BnST	bed nucleus of the stria terminalis
cDNA	complimentary DNA
CIDR	progesterone releasing intravaginal insert
CL	corpus luteum
CLIP	corticotrophin-like intermediate peptide
CNS	central nervous system
CPE	carboxypeptidase E
CV	coefficient of variance
db/db	mutant mouse model without leptin receptor
des-α-MSH	des acetylated alpha melanocyte stimulating hormone
di-α-MSH	di acetylated alpha melanocyte stimulating hormone
DMH	dorsomedial hypothalamus
E_2	estrogen
EDTA	ethylene diamine tetra acetic acid
EIA	enzyme immunoassay
ER-α	estrogen receptor α
ER-β	estrogen receptor β
FG	flurogold
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
GnRH	gonadotropin releasing hormone
GPR54	G-protein coupled receptor 54
HPLC	high performance liquid chromatography
HS014	melanocortin 4 receptor antagonist
ICV	intracerebroventricular
IIIV	third ventricle

In Situ	In situ hybridisation
Kiss1	kisspeptin
LH	luteinising hormone
LHA	lateral hypothalamus area
LSD	least significant difference
LV	lateral ventricular
MC1-R – MC5-R	melanocortin receptor 1 - 5
mRNA	messenger RNA
MTII	melanotan II – a non selective MC3-R and MC4-R agonist
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
ob/ob	mutant mouse model lacking leptin gene
Ob-Ra	short form leptin receptor
Ob-Rb	long form signaling leptin receptor
OC	optic chiasm
ORX	orexin
OVLT	organum vasculosum of the lamina terminalis
OVX	ovariectomised
OX-R1, OX-R2	orexin receptor 1 and 2
P_4	progesterone
PAM	peptidyl α-amidating mono-oxigenase
PB	phosphate buffer
PBS	phosphate buffered saline
PC1	prohormone convertase 1
PC2	prohormone convertase 2
PCR	polymerase chain reaction
Pe	periventricular nucleus
$PGF_{2\,\alpha}$	prostaglandin $F_{2\alpha}$
POA	preoptic area
POMC	proopiomelanocortin
ppORX	pre pro orexin
PR	progesterone receptor
PVN	paraventricular nucleus
PYY	peptide YY
RIA	radioimmunoassay
SEM	standard error of the mean
SHU9119	synthetic melanocortin 3 and 4 receptor antagonist
SON	supraoptic nucleus
TEA	triethanolamine aminomethane
TMB	tetramethylbenzidine
Tris	tris hydroxymethyl aminomethane
VMH	ventromedial hypothalamus
Y1 – Y6	neuropeptide Y receptors
ZI	zona Incerta
α, β, γ-MSH	α-,ß-,γ-melanocyte-stimulating hormone

β-END	β endorphin
β-LPH	β lipotrophin

Symbols

%	percent	α	alpha	µ	micro
/	per	β	Beta	±	plus or minus
<	less than	γ	gamma	º	degrees

Chapter 1
Review of the Literature

1.0 General Introduction

Reproductive function is essential to life. The production and development of gametes, transportation to a site of fertilisation, and the implantation of the fertilised gamete are an imperative aspect of female reproduction. These actions and the reproductive capabilities in the female mammal depend on a complex hormonal relationship, involving the intricate secretion of many endocrine factors, communicating between the brain, the anterior pituitary, and the gonads. Gonadotropin releasing hormone (GnRH), the reproductive gatekeeper, is produced in cells mainly located in the preoptic area (POA) of the brain. Here it is secreted into the hypophysial portal blood system to stimulate the synthesis and secretion of the gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. LH and FSH circulate to act on the ovaries stimulating secretion of the sex steroids, mainly estrogen and progesterone, which then feedback to negatively or positively regulate this axis.

The reproductive system is influenced by body condition, and negative energy balance can have a profound suppressive influence on reproductive function in the mammal. It has been proposed that a critical amount of body weight is essential for achieving and maintaining normal reproductive function in the female (Frisch 1990). Moreover, initiation of puberty is dependent on sufficient body energy stores (Kennedy and Mitra 1963), and puberty will be delayed if metabolic stores are inadequate. This is especially true in the female, whereby conditions of negative energy balance result in anovulation and infertility, a condition known as amenorrhea. The reproductive process places great demands on the body as energy is required for ovulation, conception, pregnancy and subsequent lactation. Therefore, in a teleological sense, the normal operation of the reproductive axis is

restricted to times of sufficient energy supply so that in times of negative energy balance, energy is reserved for other vital processes (eg. cellular biosynthesis, thermogenesis, locomotion etc), ensuring survival. Reproductive suppression as a result of energetic challenge can be clearly appreciated in anorexic females or elite athletes which is characterised by minimal body fat stores and amenorrhea. Anorexia nervosa is a complex psychological condition, which has etiology beyond those considered in this thesis.

The physiological evidence connecting body weight and nutritional status to reproduction is illustrious, but the central neuronal circuitry responsible for the interaction between the fat, hypothalamus, pituitary and gonads remains somewhat enigmatic. Energetic challenge results in the central inhibition of GnRH neurons and a decrease in the frequency and amplitude of GnRH secretion (I'Anson *et al.* 2000). GnRH released from the hypothalamus drives the pituitary release of LH (Clarke and Cummins 1982), therefore LH secretion is also suppressed (Henry *et al.* 2001a). The question still exists as to how GnRH neurons in the brain respond to changing levels of adiposity.

The discovery of the adipose derived hormone leptin in 1994 (Zhang *et al.* 1994), revolutionised the field of nutritional infertility. Leptin, an adipose derived hormone, was discovered to provide a fundamental link communicating with the brain to relay information regarding body energy stores. Leptin is secreted in proportion to adiposity, with normal to high leptin levels being permissive for normal GnRH and gonadotropin secretion. On the other hand, the secretion of these hormones, and the function of the reproductive axis is compromised when leptin levels are too low. Paradoxically, leptin receptors do not appear to be expressed on GnRH neurons (Quennell *et al.* 2009) and it is likely that leptin acts via one, or several interneuronal cell populations in the hypothalamus to regulate the reproductive axis. The identity of these cells remains to be elucidated, but the melanocortin family of peptides are poised to provide such a link between leptin and GnRH neurons. The melanocortins are products of the pro-opiomelanocortin (POMC) gene, and almost all POMC containing cells express the leptin receptor in the sheep (Iqbal *et al.* 2001b). Furthermore, similar to leptin, the melanocortin

peptides reduce food intake (Fan *et al.* 1997) and stimulate reproduction in the rat (Watanobe *et al.* 1999a).

Sex steroids are also prime modulators of GnRH and LH secretion, providing feedback modulation of GnRH/gonadotropin secretion. GnRH cells, however, do not express estrogen receptor-α (Lehman and Karsch 1993), again suggestive of an interneuronal pathway linking the feedback effects of estrogen on GnRH secretion. The cells that produce the melanocortins may also act as an efferent conduit for sex steroid regulation of GnRH secretion, although only a small portion of these cells express estrogen receptor-α (Lehman and Karsch 1993).

In summary, altered metabolic status and steroid milieu are relayed to the brain to regulate GnRH secretion. The understanding of how this occurs and the neuronal circuitry is continually advancing, with the melanocortins identified as notable intermediary candidates. Expounding these underlying central mechanisms will aid in the therapeutic intervention of reproductive suppression. Moreover, this may provide not only an improved understanding of the physiological processes that control health and reproduction, but may also provide potential targets for manipulation of reproductive function in humans and animals.

This thesis assessed the role of the melanocortins in the regulation of the reproductive axis in the ewe and delineated the underlying neuronal circuitry involved in transmitting leptin signals and sex steroid feedback to GnRH neurons.

1.1 Hypothalamic Amenorrhea

Excessive low body weight in the female is often correlated with reduced secretion of reproductive hormones and the cessation of menstrual cycles, a condition known as hypothalamic amenorrhea. Hypothalamic amenorrhea has been defined as the cessation of menstruation due to a dysfunction of hypothalamic signals to the pituitary gland, resulting in a failure of ovulation (Ahima 2004). Hypothalamic amenorrhea, whether it is due to excessive exercise, lowered body weight or unknown (idiopathic) factors may have serious medical consequences later in life. Prolonged amenorrhea can result in infertility and a significant loss of bone mineral density, thereby increasing risk of osteoporosis and stress fractures.

Not only are these medical consequences a burden on the individual at risk, but is also a great encumbrance on the health system. Thus, it is important to understand how loss of reproduction occurs in low body weight, and to elucidate the mechanisms involved in restoring reproduction when body weight returns to normal levels.

1.1.1 Causes

More often than not, energy deficiency is a causal factor of hypothalamic amenorrhea. Individuals of normal body weight with hypothalamic amenorrhea (eg. stress related) also have subtle defects in calorie and macronutrient uptake (Ahima 2004). It is likely that a central signal indicating insufficient energy supply is responsible for the suppression of the reproductive axis among these individuals. Those with hypothalamic amenorrhea are thought to have reduced levels of GnRH (Yen 1998), with reduced serum levels of LH, FSH and estrogen, a condition known as hypogonadotropic hypogonadism.

1.2 The estrous cycle of the ewe

The estrous cycle of the ewe is a representation of ovarian cyclicity and generally takes place over a period of 17 days (Goodman 1994) during the breeding season when ewes are sexually receptive. The estrous cycle consists of four distinct hormonal phases, being the luteal phase, the follicular phase, the pre-ovulatory surge phase, and metestrus (figure 1.0). The onset of estrus coincides with the preovulatory LH surge; both being due to the time-delayed effect of high levels of estrogen in the follicular phase of the cycle. The preovulatory LH surge is due to an outpouring of LH from the anterior pituitary, with loss of more than 90% of the LH stored in the pituitary gonadotropes (Roche *et al.* 1970). The preovulatory surge in the secretion of LH causes ovulation, being the release of an oocyte or oocytes from one or more ovarian follicles. The follicular remnants undergo physical changes, a process known as luteinisation, to form the corpus luteum (CL). During the period of CL formation the female is said to be in a state of metestrus where there is a low level of hormonal activity. Once the CL is formed the female enters the luteal phase of the cycle where the ovary prepares the female to receive and mature the conceptus, should fertilisation take place. The CL in the sheep secretes the steroid hormone progesterone which feeds back to the hypothalamus to decrease GnRH secretion. Estrogen levels are low at this stage, but act in concert with progesterone to suppress GnRH at the level of the hypothalamus, and LH and FSH at the anterior pituitary (explained in more detail in section 1.3). In the absence of fertilisation the corpus luteum lasts for 10-12 days and then regresses due to an increase of the uterine luteolytic hormone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a process known as luteolysis. As a result, progesterone concentrations fall to almost undetectable levels (Goodman 1994) and the ovarian follicles undergo a period of growth in a cycle stage known as the follicular phase. The follicular phase, a period of estrogen dominance, prepares the uterus for the receipt of spermatozoa and fertilisation of the egg. The fall in progesterone removes the inhibitory feedback to GnRH secretion, and an increase in the pulsatile secretion of GnRH causes increased pulsatile secretion of LH from the anterior pituitary. This, in turn, stimulates estrogen secretion from the follicles in the ovary, triggering a time-delayed (Clarke 1996b) GnRH/LH surge at the onset of estrus, and thus starts

the cycle over again. This is a physiological process involving the hypothalamus, anterior pituitary, and ovaries (known as the hypothalamo-pituitary-gonadal axis), with feed-forward and feedback processes characteristic of all physiological systems.

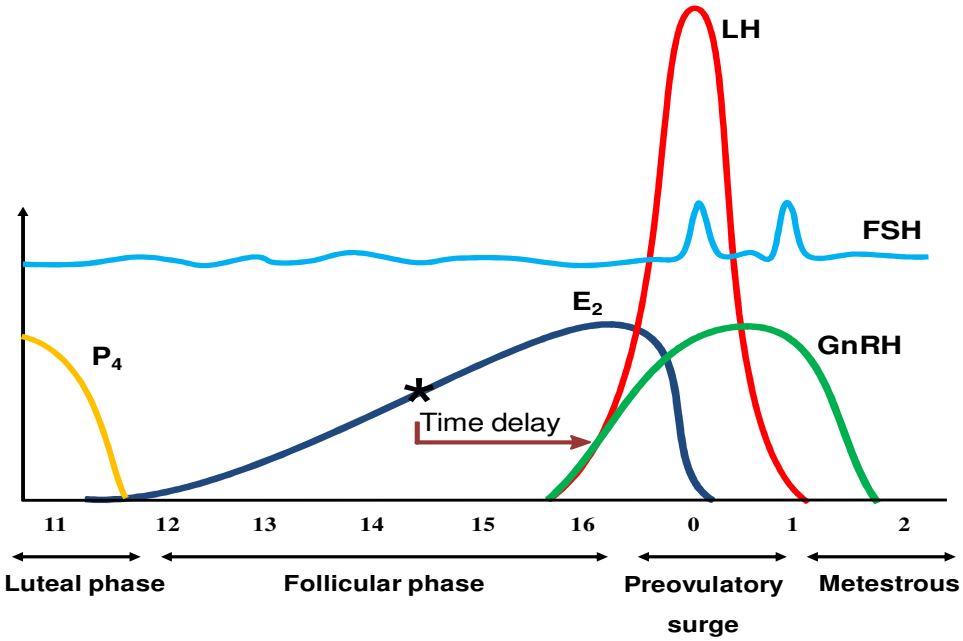

Figure 1.0: A schematic representation of the estrous cycle of the ewe. The Luteal phase is characterised by high levels of progesterone and low levels of estrogen. The follicular phase follows which in characterised by an increase in estrogen levels, reaching a threshold (*) midway through the follicular phase. This causes a time delayed increase, and peak, in GnRH and thus LH. This LH peak is known as the LH surge. Metestrous follows which is characterised by low hormonal activity. **P_4:** Progesterone **E_2:** Estrogen **GnRH:** Gonadotropin-releasing hormone **LH:** Luteinising Hormone. **FSH:** Follicle Stimulating Hormone

1.3 The hypothalamo-pituitary-gonadal axis

GnRH, a 10 amino acid peptide was the first neuropeptide identified in 1971 (Amoss *et al.* 1971; Matsuo *et al.* 1971), and the discovery won a Nobel prize for Roger Guillemin and Andrew Schally in 1977. GnRH is synthesised predominantly in a network of neurons in the POA (Lehman *et al.* 1986; Clarke and Pompolo 2005), and this neuropeptide is generally considered to be the primary brain signal responsible for the synthesis and secretion of LH and FSH from the gonadotropes of the anterior pituitary gland (Clarke 1996b). In sheep, GnRH neurons originating in the POA, project to the rostral area of the external zone of the median eminence (Polkowska *et al.* 1980; Lehman *et al.* 1986). Here it is secreted in an episodic

manner into the hypophyseal portal system to act on the gonadotropes in the anterior pituitary, and stimulates gonadotropin secretion (Clarke and Cummins 1982). LH along with FSH is released from the anterior pituitary into the peripheral circulation to allow the ovaries to secrete estrogen and progesterone (figure 1.1). LH is released into the periphery in a pulsatile manner, whereas FSH displays less pulsatility, and is thought to be regulated by other factors such as inhibin (Clarke 1996a). In sheep, GnRH and LH are released in a pulsatile concordant manner (Clarke and Cummins 1982), and every LH pulse proceeds a GnRH pulse (Clarke and Cummins 1985b). For this reason, measurement of pulsatile LH secretion is often used as a reflection of GnRH secretion. Interestingly the pattern of FSH is not tightly coupled with that of GnRH, as FSH secretion continues in the absence of pulsatile GnRH secretion (Clarke *et al.* 1978). Estrogen and progesterone are prime modulators of GnRH functioning, exerting their feedback effects on the brain and the pituitary to regulate its secretion and biosynthesis (Karsch *et al.* 1987). Point mutations in the GnRH gene causes hypogonadism (Charlton *et al.* 1983), and individuals diagnosed with Kallmann's syndrome, a disorder characterised by a defect in GnRH migration (Schwanzel-Fukuda *et al.* 1989), are infertile due to a reduction in GnRH secretion (Soules and Hammond 1980).

Throughout the luteal phase of the estrous cycle, progesterone and to a lesser extent estrogen, restrains GnRH mediated LH secretion through negative feedback actions at the level of the hypothalamus. These low levels of estrogen also exert a negative feedback action at the anterior pituitary by acting in unison with progesterone to potentiate suppression of GnRH (Figure 1.1 A). During the early follicular phase that follows, estrogen exerts negative feedback actions at the hypothalamus to suppress the synthesis and secretion of GnRH, and at the level of the pituitary to restrain gonadotrope responsiveness to GnRH (Figure 1.1 B). This negative feedback action of estrogen does not completely overcome the gonadotropin support of the ovaries and a gradual increase in estrogen levels occurs. Progesterone regulates LH pulse frequency and estrogen regulates LH pulse amplitude (Goodman and Karsch 1980), and accordingly, the frequency of GnRH and LH pulses increases with the demise of the corpus luteum (Clarke 1995a). Mid way through the follicular phase estrogen levels reach a threshold,

and unopposed by progesterone, results in a switch from estrogen negative feedback to the neuroendocrine reproductive axis, to estrogen positive feedback (Clarke 1995b) (Figure 1.1 C). The positive feedback of estrogen acts on GnRH neurons to cause a surge in GnRH secretion whereby GnRH is released in a continuous rather that episodic manner, and is also responsible for the increase in pituitary gonadotrope responsiveness to GnRH (Clarke 1995b). The increase in GnRH causes hypersecretion of LH, known as the preovulatory LH surge, and it is these two events which signify the pre-ovulatory phase of the estrous cycle and are essential for successful ovulation (Clarke 1996c). It has also been argued that the increase in GnRH pulse frequency is a causal factor of the LH surge (Clarke 1993). During a normal estrous cycle, both the fall in progesterone at luteolysis, and the follicular phase estrogen rise are required for the initiation of the LH surge and ovulation (Goodman 1994). A single challenge of estrogen to ovariectomised (OVX) ewes is capable of inducing an LH/GnRH surge (Clarke 1993) and prior progesterone treatment is able to attenuate this effect in the ewe (Karsch *et al.* 1980b; Kasa-Vubu *et al.* 1992).

It is these hormonal relationships between the hypothalamo-pituitary-gonadal axis that enable the successful completion of the estrous cycle.

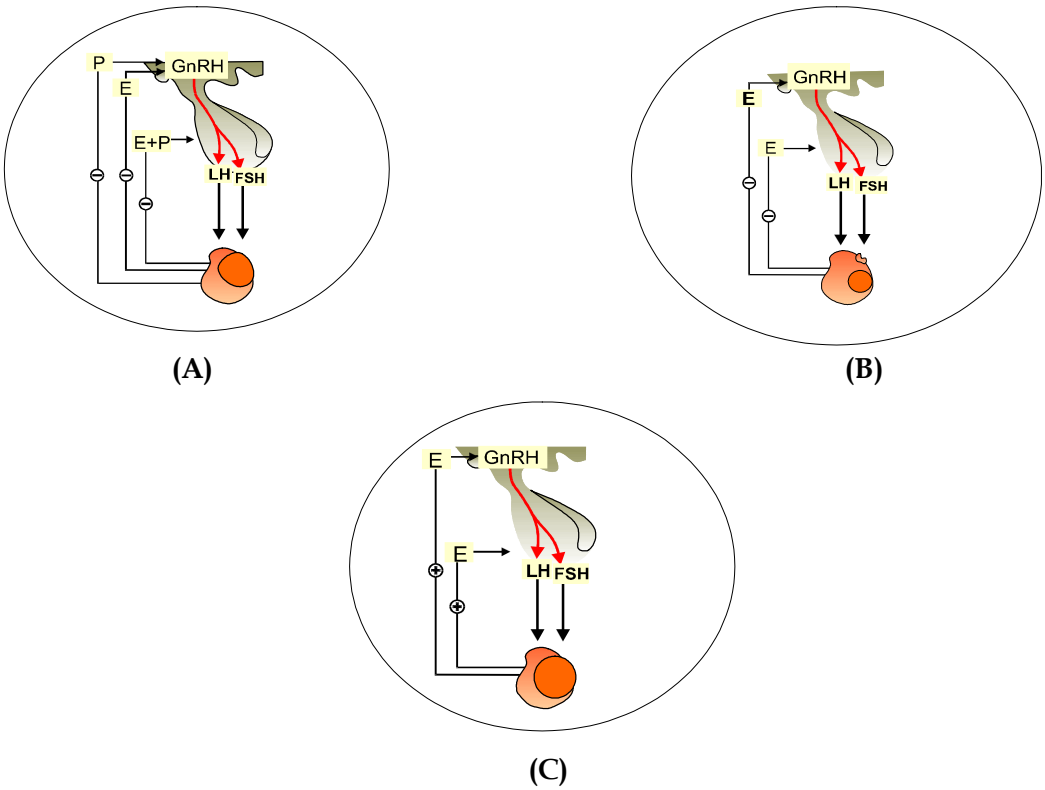

Figure 1.1: A schematic representation of the hormonal relationship between the hypothalamus, anterior pituitary and ovaries in a normal estrous cycle. GnRH secreted from the hypothalamus acts on the anterior pituitary to secrete LH and FSH which inturn allows the ovaries to secrete estrogen and progesterone. **(A)** During the luteal phase of the estrous cycle progesterone exerts a negative feedback action at the level of the hypothalamus to suppress GnRH/LH secretion. Estrogen concentrations are low during this phase, however, this hormone still exerts a negative feedback action at the hypothalamus and anterior pituitary to restrain GnRH and LH secretion by acting in unison with progesterone. Progesterone alone has no effect on the pituitary, but potentiates the effects of estrogen. **(B)** During the follicular phase of the estrous cycle estrogen exerts negative feedback actions at the hypothalamus and anterior pituitary, thus decreasing GnRH and LH. **(C)** Midway through the follicular phase, there is a neuroendocrine switch from negative to positive feedback by estrogen on the hypothalamus and anterior pituitary, causing a GnRH/LH surge which initiates the preovulatory LH surge and ovulation. Modified from (Clarke 1996b)

1.3.1 Central GnRH distribution

GnRH neurons are diffusely located throughout the POA and medial basal hypothalamus of the brain, with the most abundant population located in the medial POA in both the rodent (Witkin *et al.* 1982) and ovine (Polkowska *et al.* 1980) species. In the ewe, occasional GnRH cells are also found in the bed nucleus of the stria terminalis (BnST), the perifornical area (PFA), the supraoptic nucleus and the parventricular nucleus (PVN), with relatively few cells in the arcuate nucleus (ARC) or the ventromedial nucleus (VMN) (Lehman *et al.* 1986). GnRH neurons originating in the POA project to the median eminence where GnRH is stored in the external zone prior to release into the hypophyseal portal vessels (Samson *et al.* 1980). GnRH neurons also project to the organum vasculosum (OVLT) (Samson *et al.* 1980), but the function of this projection remains unclear.

1.3.1.1 Pulsatile LH secretion reflects GnRH secretion

In 1982 Clarke and Cummins described a method allowing collection of hypophyseal portal blood from conscious sheep. This enabled the measurement of GnRH and LH, revealing the pulsatile nature of GnRH secretion and how the secretion of GnRH drives LH secretion (Clarke and Cummins 1982). Specifically, 97% of LH pulses were seen to be concomitant with a pulse of GnRH, and it was proposed that the frequency pattern of LH secretion measured, reflected the frequency pattern of GnRH secretion. A parallel pattern of occurrence between LH and GnRH amplitude and duration however does not hold true. A reduction in LH amplitude may indeed mirror a reduction in GnRH pulse amplitude, however it is also possible that a decline in LH pulse amplitude is a causal effect of reduced responsiveness at the anterior pituitary gland to GnRH (due to the feedback effects of steroids on gonadotropes). There is, however, a strong inverse relationship between GnRH pulse frequency and LH pulse amplitude, such that as the former is decreased when the latter is increased (Clarke and Cummins 1985a). This substantially explains the changes in LH pulse frequency and amplitude that occur in the transition from the luteal to the follicular phase of the cycle.

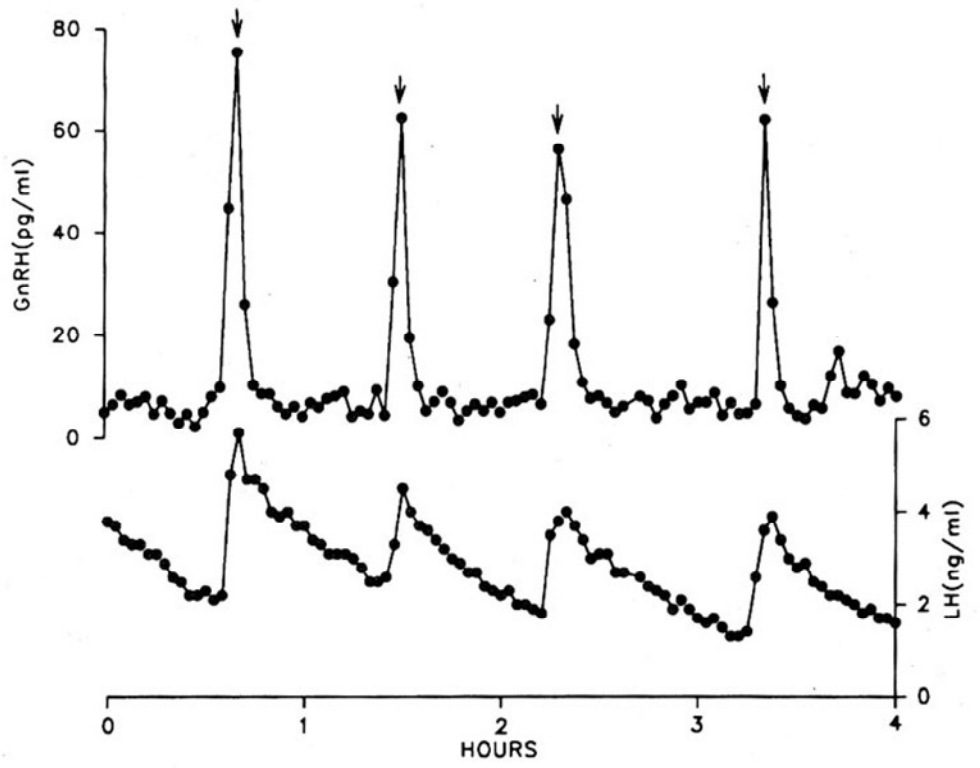

Figure 1.2: A graphical representation of the parallel relationship between pulsatile GnRH secretion from the hypophyseal portal blood and pulsatile LH secretion from jugular blood. Arrows indicate a GnRH pulse. Adapted from (Clarke and Cummins 1982).

1.3.1.2 Ovarian sex steroids communicate with GnRH cells via an interneuronal pathway

It is unequivocal that the sex steroids, estrogen and progesterone, coordinate the changes in GnRH and LH secretion via negative and positive feedback mechanisms. In recent years, dual label immunocytochemistry has revealed positive staining for estrogen receptor beta (ER-β) on GnRH neurons in the POA of the rat (Hrabovszky *et al.* 2001). This revelation led to the notion of a direct effect of estrogen on GnRH neurons, and was further supported in the rat by the observation that a maximum of 20% of GnRH neurons contain ER-β mRNA (Herbison and Pape 2001). Nevertheless, it appears that the beta sub-type of estrogen receptor plays a minor role in the feedback control of GnRH neurons (Krege *et al.* 1998). There have been no reports of estrogen receptor alpha (ER-α) (Shivers *et al.* 1983; Herbison and Theodosis 1992) or progesterone receptor (PR) (Skinner *et al.* 2001) expression on GnRH neurons, and it has been suggested that these sex steroids exert their effects

via intermediary neurons that possess the relevant sex steroid receptors. The exact nature of such an interneuronal pathway that permits the feedback effects of sex steroids appears to be complex and has not been fully deciphered. The discovery of kisspeptin in the mammalian brain, and the role that this plays in mediating feedback effects of steroids was a seminal advance in the understanding of sex steroid feedback to GnRH neurons. This is discussed in section 1.10.2.

1.4 Seasonality

Sheep are seasonal breeders and utilise photoperiod to regulate their circannual neuroendocrine reproductive breeding pattern (Lincoln 1979; Thiery *et al.* 2002). During long days in spring and summer tonic GnRH/LH secretion is suppressed in a steroid dependent (Karsch *et al.* 1993) and steroid independent (Robinson *et al.* 1985) manner. Reproductive function resumes in autumn and winter when day length is shortened (Yeates 1949). The duration of nocturnal melatonin secretion appears to be the major factor contributing to the season regulation of reproductive patterns in the ewe (Bittman *et al.* 1983), increasing responsiveness to estrogen negative feedback in long days and thereby suppressing LH release (Karsch *et al.* 1980a).

1.5 Negative energy balance and the reproductive axis

In 1963, Kennedy and Mitta observed that the timing of puberty is predicted more precisely by body weight rather than chronological age (Kennedy and Mitra 1963), and later in 1980, Frisch proposed that the level of body fat could trigger the initiation of reproductive function (Frisch 1980). Since these early studies a great deal of work has implicated the importance of sufficient adiposity to successful reproduction. Insufficient energy stores delays the onset of sexual maturation (Foster and Olster 1985), and in sexually mature animals, energy balance and body weight must be maintained within an acceptable physiological range in order for the body to be capable of reproducing. Inadequate food intake or an increase in energy demand, without a matched increase in caloric consumption, can lead to fertility problems in a wide range of species.

Metabolic challenge leads to inhibition of the hypothalamo-pituitary-gonadal axis at all levels, but the major cause of reproductive suppression is a decrease in the pulsatile secretion of GnRH, with consequent reduction in pulsatile secretion of LH and sex steroids. This nutritional suppression of GnRH and LH release has been observed in the rat (Cagampang *et al.* 1990), monkey (Cameron and Nosbisch 1991), sheep (Foster *et al.* 1989; Tatman *et al.* 1990) and human (Pirke *et al.* 1989). Body weight, however, must drop below a critical level before the nutritional suppression of LH occurs (Tatman *et al.* 1990), indicating that a minimal amount of body weight is required for reproductive success. This inhibition of the reproductive axis is due, in part, to an increase in the sensitivity to the negative feedback effects of sex steroids, but a steroid-independent suppression also exists (Foster and Olster 1985).

Multiple metabolic factors signal to the brain to collectively regulate the reproductive neuroendocrine system, including glucose, insulin and leptin (Schneider 2004). The discovery of the peripheral adipostatic hormone leptin 15 years ago sparked great interest and created a rapid restructure in the way we think about the link between nutrition and reproduction.

1.6 Sex steroids and energy balance

Gonadal steroids not only regulate reproductive function, but also influence food intake and body weight. Daily food intake in women has been found to be lowest during the peri-ovulatory phase when estrogen concentrations are at their highest (Lissner *et al.* 1988; Buffenstein *et al.* 1995), and estrogen deficiency increases food intake and weight gain, whilst estrogen treatment reverses this effect (Asarian and Geary 2002). Furthermore, estrogen receptors are located in areas of the brain known to coordinate metabolism such as the ARC, and the VMH (Diano *et al.* 1998) and estrogen treatment directly into either of these areas or in the PVN reduces food intake and body weight (Butera and Czaja 1984; Palmer and Gray 1986). ER-α, but not ER-β knockout mice exhibit an obese phenotype (Heine *et al.* 2000; Naaz *et al.* 2002), suggestive of a requirement for the former in the modulation of energy homeostasis.

POMC and neuropeptide Y (NPY) neurons of the ARC are two primary neurons believed to transmit sex steroid information in the brain to modulate energy balance, which may indeed be the case, however only 10-20% of these neurons express ER-α (Lehman and Karsch 1993; Skinner and Herbison 1997). Thus, the enigma persists as to how sex steroids not only regulate reproductive function but also energy balance.

1.7 Leptin

1.7.1 Discovery and physiological functions

Leptin, first discovered in 1994 (Zhang *et al.* 1994), is a 167 amino acid protein and a product of the *ob* gene produced from adipocytes. Leptin was first discovered to reduce body weight (Zhang *et al.* 1994) and the name was thus derived from the Greek word leptos, meaning thin. Since its discovery, leptin has been revealed to play a critical role in signalling information regarding energy stores and calorie consumption peripherally to brain centres that regulate ingestive behaviour, thermogenesis and reproduction (Friedman and Halaas 1998; Clarke *et al.* 2001). Leptin from fat is secreted into the bloodstream in proportion to adiposity in humans and is positively correlated with body fat mass (Considine *et al.* 1996). In a state of caloric restriction leptin levels fall rapidly (Flier 1998).

In humans, a homozygous mutation in the leptin receptor gene (Clement *et al.* 1998) or congenital leptin deficiency (Montague *et al.* 1997) results in morbid obesity, associated with impaired reproductive function. A similar phenotype is displayed in the rodent with a point mutation in the leptin receptor gene (Chen *et al.* 1996). Human obesity however, is generally not associated with a leptin defect or deficiency, rather an insensitivity of leptin, which means that the hormone no longer negatively regulates food intake (Considine *et al.* 1996). The observation in both humans and animals, that any deficiency in the leptin system results in morbid obesity and infertility, reinforces the imperative actions of leptin in energy and reproductive homeostasis.

A popular model in which leptin exerts its effect in the brain is first by primary response cells in the ARC. Such cells are those which express POMC and NPY which

both express leptin receptors in the sheep (Iqbal *et al.* 2001b), as in other species (Cheung *et al.* 1997). It is hypothesised that these first order neurons then project to second order neurons in other areas of the brain, which then exert metabolic and reproductive effects (Schwartz *et al.* 2000).

1.7.2 Leptin and leptin receptor expression

There are six splice variants of leptin receptors, but the most commonly studied forms are Ob-Ra and Ob-Rb referred to as short and long receptor forms respectively. Leptin receptors have been found in several brain regions including the cortex, thalamus and hippocampus in both the rodent (Mercer *et al.* 1996; Bennett *et al.* 1998; Elmquist *et al.* 1998a) and sheep (Dyer *et al.* 1997), with a strong expression in the hypothalamic ARC, DMH, PVN, VMN and the lateral hypothalamus (LHA) in the mouse (Mercer *et al.* 1996) and sheep (Iqbal *et al.* 2001b). Additionally, leptin receptors have also been reported in the brain stem and pituitary gland of the sheep and cow (Dyer *et al.* 1997; Chelikani *et al.* 2003). Peripherally, leptin receptors are expressed in the testes and the accessory reproductive tract of the rodent and the anterior pituitary and adipose tissue in the sheep (Dyer *et al.* 1997).

Ob-Ra, the 'short form' leptin receptor is present in the choroid plexus and in the microvessels of the brain (Tartaglia *et al.* 1995; Bjorbaek *et al.* 1998), and is thought to be involved in clearance and/or transport of leptin from the periphery to the brain across the blood brain barrier (Tartaglia *et al.* 1995; Golden *et al.* 1997). This is evidenced in rats where leptin transport is decreased in animals with point mutations in the short form leptin receptor (Kastin *et al.* 1999).

There is good evidence that the 'long form' leptin receptor, which is highly abundant in the hypothalamus, is the only leptin receptor capable of signal transduction (Lee *et al.* 1996a). This suggests that a key responsibility of these hypothalamic receptors is to relay the neuroendocrine effects of leptin, even though other brain regions are also targeted. Importantly, these receptors co-localise with numerous neuropeptides which are responsible for the control of

both food intake and reproduction, and leptin either stimulates or inhibits these neurons (Elmquist *et al.* 1998b; Iqbal *et al.* 2001b).

1.7.3 Leptin and metabolic homeostasis

Following leptin treatment to sheep (Henry *et al.* 1999), pigs (Barb *et al.* 1998) or rodents (Halaas *et al.* 1995) voluntary food intake is reduced. Additionally, peripheral leptin concentrations alter the expression of many ovine appetite regulating genes (Henry *et al.* 2000; Henry *et al.* 2001b). Moreover, mutations in the leptin or leptin receptor gene causes obesity in humans (Clement *et al.* 1998) and mice (Bray and York 1979), nevertheless, such mutations are rare and do not account for the increasing prevalence of obesity. Treatment with recombinant leptin to a child with a mutation of the *ob* gene caused weight loss just 2 weeks post therapy and continued over 12 months (Farooqi *et al.* 1999). On the other hand, leptin treatment to adults with diet-induced obesity produced disappointing results with only modest reductions in body weight over a 12 week period (Heymsfield *et al.* 1999), leading to the belief that obesity is associated with a leptin resistant state.

Fasting reduces plasma leptin levels in rodents (Ahima *et al.* 1996), sheep (Henry *et al.* 2000; Henry *et al.* 2001a) and humans (Weigle *et al.* 1997) without any reduction in body weight, presumably ensuring appetite drive remains high until body weight is corrected. Fasting has no effect on leptin receptor mRNA in the hypothalamus of rats (Bennett *et al.* 1998), however expression of the long form leptin receptor in the ARC increases following long term food restriction in the ewe (Dyer *et al.* 1997), suggestive of enhanced leptin sensitivity to neuroendocrine centres.

1.7.4 Leptin and reproduction

One of the first indications that leptin may influence reproductive function was observed from the infertile phenotype of the o*b/ob* and *db/db* mouse which respectively lack either a functional leptin gene or the mutated leptin receptor gene (Ingalls *et al.* 1950). This notion gained further credence in 1996 and 1997 with observations that leptin treatment to *ob/ob* mice (Barash *et al.* 1996; Chehab

et al. 1996) or human males (Mounzih *et al.* 1997) genetically deficient of leptin restores reproductive function back to normal. Additionally, leptin levels below 3mg/ml and adiposity below 15% in female humans is likely to be associated with menstruation disorders and impaired reproductive function (Tataranni *et al.* 1997).

Analogous to the leptin deficient state of the *ob/ob* mouse, leptin levels fall in food-restricted animals concomitant with a reduction in LH secretion. Central or peripheral treatment of these animals with leptin restores pulsatile LH release and normal ovarian cycles (Ahima *et al.* 1996; Henry *et al.* 2001a). Moreover, leptin restores reproductive function if administered to women with hypothalamic amenorrhea that is either exercise-induced or due to low body weight (Welt *et al.* 2004). Thus, it seems apparent that leptin is vital for normal reproductive function, and the fall in leptin levels, that is characteristic of energy deficiency, is a causative element to the impaired reproductive state.

In the normally fed rat (Watanobe 2002) or sheep (Henry *et al.* 1999), leptin reduces food intake but has no effect on LH release. This suggests that the physiological concentration of leptin in a normally fed animal may already be at maximal concentrations by which leptin can exert a stimulatory effect on the neuroendocrine reproductive axis, and leptin in excess of this is without effect. In contrast, leptin treatment to long-term food-restricted sheep increases pulsatile LH secretion, but has no effect on food intake (Henry *et al.* 2001a), suggesting that leptin is unable to overcome the hunger drive that persists in the undernourished animal.

Although it is clear that leptin can restore gonadotropic function in lean or food-restricted animals, the neuronal pathways involved remain elusive. Some studies have suggested a direct influence of leptin on GnRH neurons after immortalised GnRH GT1-7 neurons were found to express the leptin receptor (Zamorano *et al.* 1997; Magni *et al.* 1999). However, alternative observations in the rodent (Hakansson *et al.* 1998; Quennell *et al.* 2009) and monkey (Finn *et al.* 1998) report that leptin receptors are not expressed on GnRH neurons *in vivo*. Moreover, mice with GnRH cell-specific leptin receptor knockout display a normal reproductive phenotype (Quennell *et al.* 2009).

Converging lines of evidence propose that leptin acts within the hypothalamus of the brain, and more specifically within the ARC, to indirectly influence GnRH secretion. Several ARC neuropeptides, such as POMC and NPY that have the dual function of regulating appetite and reproductive function express the long form leptin receptor (Cheung *et al.* 1997; Iqbal *et al.* 2001b), placing leptin and its receptor in an ideal position to serve as conduit between fat stores and the brain to regulate GnRH. It is important to note, that simply re-feeding fasted animals is capable of restoring GnRH and LH secretion prior to an increase in leptin levels (Szymanski *et al.* 2007), and is suggestive that the pathways involved in the metabolic regulation of fertility are not only limited to leptin signalling.

Collectively, the research to date suggests that leptin exerts its neuroendocrine reproductive effects via an interneuronal pathway within the brain, however the complete architecture of this system remains to be delineated.

1.8 Appetite regulating peptides

The central regulation of appetite involves the intricate coordination of many neuropeptides and neurotransmitters. The hypothalamus is considered the central feeding hub where many orexigenic and anorectic neuropeptides are synthesised and released. Many of these appetite regulating peptides also regulate aspects of the reproductive system. POMC, kisspeptin, Orexin (ORX) and NPY are all examples of peptides with such a dual function.

1.9 Pro-opiomelanocortin (POMC)

The pleiotropic POMC prohormone is a particularly interesting molecule as it produces a number of post-translational peptides which elicit opposing physiological effects. The POMC gene encodes the melanocortins that reduce food intake (Fan *et al.* 1997) and stimulate reproduction (Watanobe *et al.* 1999a). The same gene also encodes the endogenous opioid, β-endorphin (β-END), which negatively regulates reproduction (Bonavera *et al.* 1993) and stimulates food intake (Grandison and Guidotti 1977).

The POMC gene is expressed in the pituitary gland, skin, immune system and brain (Yeo *et al.* 2000) and is cleaved in a tissue specific manner to produce ACTH, the

melanocortins (α, β, and γ melanocyte stimulating hormone; MSH), β-END, and β-lipotrophin (β-LPH) (See section 1.9.2 for further detail on POMC post-translational processing). POMC producing peptides have established roles in skin pigmentation, adrenal steroidogenesis, memory, behaviour, inflammation, pyretic control, pain perception, blood pressure, nerve growth, and most importantly for the purpose of this thesis, metabolic and reproductive control. Humans with rare mutations in the POMC gene have red hair pigmentation, pale skin, adrenal insufficiency and early onset of obesity (Krude *et al.* 1998; Krude *et al.* 2003). Observations in these individuals however are limited due to the young age and small number of patients, and other physiological disorders, such as reproductive defects may reveal themselves over the coming years.

Most POMC-expressing cells of the ARC contain leptin receptors, as shown in rodents (Cheung *et al.* 1997), sheep (Iqbal *et al.* 2001b) and monkeys (Finn *et al.* 1998). This evidence collectively gives rise to the implication of POMC or its precursors to mediate the effects of leptin in the hypothalamus. POMC mRNA expression is decreased in food-restricted rats and leptin treatment is able to attenuate this effect (Korner *et al.* 2001). Expression of the POMC gene has also been shown to be reduced by chronic food restriction in sheep (McShane *et al.* 1993), although this effect has not always been seen (Henry *et al.* 2000). Furthermore, expression of the melanocortin receptors, MC-3 and MC-4 (Iqbal *et al.* 2001a), does not change with altered body weights, reflected in leptin status. In other work, there was no change in the expression of POMC mRNA across the estrous cycle of the ewe (Walsh *et al.* 1998), thereby questioning whether gonadal steroids have a major effect on these cells. Nevertheless, this does not preclude a change in post-translational POMC derived peptides.

A substantial body of research does indeed implicate the POMC gene and its derivatives to be prime regulators of reproduction in times of negative energy balance. The ratio of these stimulatory and inhibitory POMC products is extremely important as it greatly affects the reproductive outcome, however little is known about the way POMC is transcribed under differing body weights, or sex steroid milieu. Because the POMC gene is post-translationally processed into a

number of peptides, the possibility of regulation beyond the point of transcription is quite realistic; indeed, this is a major focus of this thesis.

1.9.1 Central distribution of POMC

The POMC gene is expressed in the ARC of the hypothalamus, the pituitary, in the nucleus of the solitary tract (NTS) and in several peripheral tissues in the rat (Jacobowitz and O'Donohue 1978). These POMC neurons are found specifically in both the medial and ventral ARC, with a prominent distribution expressed in the lateral ARC (Cowley *et al.* 2001). A population of POMC containing cells are also found in the pars distalis of the pituitary, where adrenocorticotropin (ACTH) and β-lipotropin are synthesised and released from the corticotrophs. In the hypothalamus and the neurointermediate lobe of the pituitary, POMC is more extensively processed to produce the melanocortins and β-END (discussed in more detail below; see section 1.9.2). In the brain of the sheep POMC mRNA is found only in the ARC with no expression in the NTS (IJ Clarke and J Iqbal, unpublished observation). POMC-containing neurons project to the septum, the nucleus interstitialis stria terminalis, the medial POA, DMH, and periventricular nuclei (Pe), with moderate number of fibres observed in the PVN and ARC, the amygdala, the NTS, the mammillary body, the central grey matter, the cuneiform nucleus, and the nucleus of the solitary tract in the rat (Jacobowitz and O'Donohue 1978). Recent studies in sheep have also reported POMC projections to the VMN and Pe (Qi *et al.* 2008; Qi *et al.* 2009a).

1.9.2 Post-translational processing

Many neuropeptides are initially present as biologically inactive prohormones requiring several proteolytic steps and post-translational processing in order to produce a diversity of biological peptides. Neuroendocrine prohormone convertase 1 (PC1; also known as PC3) and 2 (PC2) are believed to be responsible for the processing of many neuropeptide precursors in humans (Miller *et al.* 2003; Pan *et al.* 2005). In particular PC1 and PC2 have been shown to cleave the POMC prohormone at paired basic residues to produce several biological peptides which are presumably co-stored and co-released (Wilkinson 2006). Post-translational processing of POMC is tissue specific (Smith and Funder 1988; Pritchard *et al.* 2002)

and has been described in the pars distalis of the pituitary, the neurointermediate lobe of the pituitary and in the ARC of the hypothalamus (See Figure 1.3 for POMC processing in the hypothalamus). PC1 processes the neuropeptide precursors into higher molecular weight peptides while PC2 is more efficient in producing smaller peptides (Pan *et al.* 2005). Within the ARC and the neurointermediate lobe, POMC is extensively processed to the melanocortins and β-END. PC1 first cleaves POMC into the large pro-adrenocorticotropin (pro-ACTH) and β-LPH molecules. ProACTH is further cleaved again by PC1 to generate a 16kDa N-terminal peptide, joining peptide and ACTH. PC2 then cleaves all ACTH to generate $ACTH_{1-17}$ and corticotrophin-like intermediate peptide (CLIP). PC2 also cleaves β-LPH to generate γ-LPH and β-END and γ-MSH is produced via PC2 cleavage of the N-terminal. In sheep and humans, but not rats γ-LPH is cleaved by PC2 to generate β-MSH (Pritchard *et al.* 2002). Carboxypeptidase E (CPE) 'trims' the c-terminal basic amino acids from all $ACTH_{1-17,}$ and peptidyl α-amidating mono-oxigenase (PAM) enzyme amidates the peptide to produce des-acetyl α-MSH (des-α-MSH) and enable bio-activity. Finally, the N-terminus of the melanocortins and β-END may be acetylated by an N-acetyltrasferase (see section 1.9.2.1 for more detail). Processing follows a similar pattern in the pars distalis of the pituitary, however in the human at least, PC2 is not expressed in this gland and therefore α-MSH is not produced and the main POMC derived products are N-terminal peptide, ACTH, and β-LPH (Smith and Funder 1988; White and Gibson 1998).

1.9.2.1 Amidation and acetylation

POMC derived peptides are amidated and/or acetylated, the former generally being required for biological activity, and the latter alters the potency and stability of the peptide. Acetylation of the N-terminal serine residue increases the ability of α-MSH to reduce food intake, but acetylation of β-END nullifies biological activity (Tsujii and Bray 1989; Abbott *et al.* 2000). The enzyme(s) (acetylase) responsible for this process have not been identified (Pritchard *et al.* 2002).

Acetylated α-MSH levels are reduced in leptin deficient *ob/ob* mice, increased in leptin-treated *ob/ob* mice, and leptin rapidly induces the enzymatic activity of an unidentified N-acetyltransferase (measured by *in vitro* activity) in POMC neurons

(Guo *et al.* 2004). Moreover, acetylated α-MSH is much more stable and resistant to peptidase degradation compared to des-α-MSH (Guo *et al.* 2004). This acetylation process likely occurs *en passage* to terminal beds (Bunel *et al.* 1990), or in secretory vesicles just prior to exocytosis in the intermediate lobe of the pituitary (Millington *et al.* 1986), and acts on POMC efferent neurons to regulate many neuroendocrine functions. Due to the opposing actions that POMC-derived hormones can have on both energy intake and reproductive function, and the increase or decrease in potency that acetylation can have on α-MSH and β-END respectively, it is important to delineate these post-translational products under varying reproductive conditions. Therefore the post-translational products of POMC cells, in particular the degree of acetylation of these peptides may determine the action on GnRH cells.

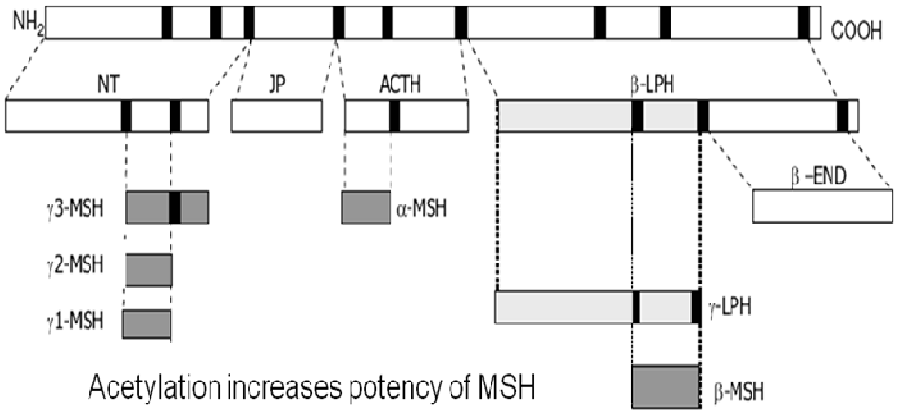

Figure 1.3: A schematic representation of the post-translational processing of the POMC gene in the hypothalamus. The POMC gene is transcribed to produce a range of products including β-lipotrophin (β-LPH) and adrenocorticotrophin (ACTH). These products are further cleaved to produce the melanocortins, α-, β- and γ-melanocyte stimulating hormone (α-,β- and γ-MSH), and the endogenous opioid β-endorphin (β-END). Further acetylation increases the potency of α-MSH, but renders β-END inactive.

1.9.3 Sex steroids and POMC

Approximately 20% of POMC neurons in the ARC possess sex steroid receptors (Dufourny *et al.* 2005) and rudimentary evidence demonstrates both estrogen and progesterone regulates POMC mRNA expression in some species. In the rat, POMC mRNA expression is reduced at the time of the pre-ovulatory LH surge (Wise *et al.* 1990; Bohler *et al.* 1991), and treating OVX rats with estrogen results in decreased POMC mRNA expression (Wilcox and Roberts 1985; Tong *et al.* 1990; Petersen *et al.* 1993). Furthermore, progesterone treatment is able to attenuate or reverse this effect (Wilcox and Roberts 1985; Wise *et al.* 1990). These studies have all been predicated on the belief that β-END negatively regulates GnRH cells and that a reduction in expression of the POMC precursor gene reduces opioid tone at the time of the preovulatory GnRH/LH surge.

Conversely, others have suggested that sex steroids have no effect on these cells after no change in POMC mRNA expression was evident in the ARC of the ewe during the estrous cycle (Walsh *et al.* 1998). In addition, total α-MSH protein levels in the mediobasal hypothalamus and POA of the rat brain did not change across the estrous cycle (Khorram *et al.* 1985). Further corroborating the null effect of sex steroids on these cells, is the finding that a reduction in POMC mRNA is not essential for the LH surge in OVX rats treated with estrogen and progesterone; a robust LH surge occurred in this model without a change in POMC mRNA expression (Petersen *et al.* 1993).

Sex steroid effects on post-translational processing of POMC remain to be determined and differential processing of the POMC precursor peptide could occur. In other words, sex steroids could alter the activity of the post-translational enzyme gene expression and the activity of these enzymes. Additionally, indirect interneuronal effects are also possible as synaptic input to POMC cells are increased by estrogen treatment (Gao *et al.* 2007).

1.9.4 β-endorphin (β-END)

β-END is post-translationally processed into 6 derivatives within the hypothalamus, which are all structurally similar, yet elicit very distinct biological effects (Millington and Smith 1991). β-END_{1-31} is the primary form found in the human and rat hypothalamus and is a potent opioid receptor agonist (Millington and Smith 1991). β-END_{1-31} is sequentially processed to produce the opioid antagonist β-END_{1-27} and β-END_{1-26} which along with the alpha-N-acetyl form of all three derivatives lacks opioid receptor activity (Tsujii and Bray 1989). The β-END peptides, together with other opioid peptides, enkephalins and dynorphin, act on the μ- and κ-receptors (Grandison and Guidotti 1977; Kieffer 1999). In contrast to the agonistic melanocortins, the endogenous opioid β-END typically increases food intake (Grandison and Guidotti 1977) and inhibits the reproductive axis (Bonavera *et al.* 1993).

1.9.4.1 β-END and food intake

Central administration of β-END increases food intake in rodents (Grandison and Guidotti 1977; Tsujii and Bray 1989) and sheep (Baile *et al.* 1987). Additionally, plasma β-END concentrations are increased in over weight and obese humans compared to normal weight subjects, and is positively correlated with percentage body fat (Obuchowicz and Obuchowicz 1997).

1.9.4.2 β-END and reproduction

Opioids, in particular, β-END, consistently exerts a tonic inhibitory tone over pulsatile LH secretion in rats (Leadem and Kalra 1985; Bonavera *et al.* 1993), monkeys (Gilbeau *et al.* 1985), sheep (Horton *et al.* 1989) and humans (Quigley and Yen 1980). It is thought that the β-END inhibitory tone is especially relevant during the luteal phase of the estrous cycle to mediate the negative feedback effects of progesterone on GnRH neurons (Horton *et al.* 1989; Behrens *et al.* 1993). The opioid antagonist naloxone is unable to stimulate LH secretion in food restricted lambs (Recabarren *et al.* 1990), suggesting that opioid modulation is absent in times of negative energy balance.

1.9.5 Melanocortins

The melanocortins are a family of peptide hormones which are derived by post-translational cleavage of the POMC precursor molecule and include ACTH, and α, β, and γ-MSH (figure 1.3). In addition, the melanocortin system also includes the endogenous inverse agonists, agouti and agouti related peptide (AGRP), 5 G-protein coupled receptors, and the melanocortin receptors 1-5 (MC1-R-MC5-R). Within the brain, AGRP is produced in the same cells of the ARC that produce NPY (Hahn *et al.* 1998), and AGRP acts as an inverse agonist at melanocortin receptors MC3-R and MC4-R (Fong *et al.* 1997; Ollmann *et al.* 1997). β-MSH is not produced in the rat (Pritchard *et al.* 2002), and γ-MSH is thought to be more important in cardiovascular function (Humphreys 2007), and has higher affinity for MCR-3 than MC4-R (Abbott *et al.* 2000). α-MSH, therefore, is the most widely studied of the melanocortins.

1.9.5.1 Melanocortin receptors

The melanocortin receptors are a family of 5 G-protein coupled receptors (MC1-R-MC5-R), all of which have varying physiological functions. Melanocortins activate the MC1-R, MC2-R, and MC5-R in the hypothalamus to regulate skin pigmentation, adrenal steroidgenesis and thermoregulation (Chakraborty *et al.* 1999; Schioth and Watanobe 2002). Of the 5 melanocortin receptors, MC3-R and MC4-R are the most abundantly expressed within the brain and are the key melanocortin receptors involved in food intake and reproductive function. The MC3-R is located on neurons in the ARC, and the MC4-R has more widespread expression throughout the brain, within the cortex, thalamus, hypothalamus, brainstem and spinal cord of the rat (Mountjoy *et al.* 1994). The receptors display a similar distribution in sheep, with both the MC3-R and MC4-R found in the septum, medial POA and LHA regions of the brain (Iqbal *et al.* 2001a), and MC3-R, but not MC4-R expression, is expressed in the ARC (Iqbal *et al.* 2001a). MC4-R appears to be the primary melanocortin receptor involved in the pathways that controls energy balance (Yeo *et al.* 2000) and reproductive function (Watanobe *et al.* 1999a). The exact role for the MC3-R remains somewhat unclear. It has been proposed that this receptor subtype may have a regulatory role in energy metabolism and food

intake (Lee *et al.* 2007; Lee *et al.* 2008), however others have shown that treatment with a MC3-R agonist is without effect on food intake in male Wistar rats (Kask *et al.* 2000). A role for the MC3-R in reproductive function appears ambiguous after one group reported that a non-selective MC3-R/MC4-R agonist, but not a selective MC3-R agonist, was able to induce the pre-ovulatory prolactin surge in the rat (Watanobe *et al.* 2001). Conversely, others report that MC3-R agonist administration directly into the POA of rats is capable of stimulating pulsatile LH release, and treatment of immortalised GT-1 cells with this agonist stimulated GnRH secretion (Stanley *et al.* 2003).

Although research into the role of the melanocortins in nutritional infertility has predominantly focussed on α-MSH, there is an increasing body of literature suggesting a critical role for β-MSH. Like α-MSH, β-MSH is also capable of activating the MC4-R, with one study suggesting a higher affinity of β-MSH to MC4-R than that of α-MSH (Harrold *et al.* 2003). This thesis focuses on the role of the most widely studied melanocortin, α-MSH.

1.9.5.2 Leptin and melanocortins

POMC neurons are considered a first order neuronal target for leptin action. The long form leptin receptor is expressed by the majority of ARC POMC neurons in both rodents (Cheung *et al.* 1997) and sheep (Iqbal *et al.* 2001b), consistent with the fact that leptin selectively stimulates these cells (Cowley *et al.* 2001). Leptin rapidly causes the release of α-MSH from rat hypothalami (Kim *et al.* 2000) and leptin infusion into the median eminence or POA of starved rats, but not normal weight rats, increases GnRH, LH and α-MSH secretion (Watanobe 2002). Others have also shown that leptin treatment to normally fed male rats increases total α-MSH levels in the ARC, as well as acetylation of this peptide (Guo *et al.* 2004). Conversely, leptin stimulation of α-MSH is not operative in diet-induced obese animals that are insensitive to the effects of leptin (Enriori *et al.* 2007). POMC mRNA is down regulated in rodents with reduced leptin signalling as seen in the *ob/ob* and *db/db* mouse and in fasting, and leptin treatment can ameliorate POMC expression back to levels seen in wild-type controls (Schwartz *et al.* 1997; Thornton *et al.* 1997). Both leptin and melanocortin agonists have been found to have

similar actions in lowering body weight and food intake in normal or overweight rats (Cettour-Rose and Rohner-Jeanrenaud 2002). Furthermore these two factors are both capable of activating the reproductive axis by stimulating pulsatile LH release (Ahima *et al.* 1996; Limone *et al.* 1997) and restoring the LH surge (Kohsaka *et al.* 1999; Watanobe *et al.* 1999b) in undernourished rats.

1.9.5.3 Melanocortins and food intake

The first suggestion of the melanocortin systems involvement with energy homeostasis came about when icv administration of α-MSH to the rodent resulted in a suppression of food consumption (Panskepp *et al.* 1976). Since this time, countless studies have been carried out which support the assertion that α-MSH is a negative regulator of energy balance. Both α-MSH and β-MSH have been shown to reduce food intake via the MC4-R (Kask *et al.* 2000) and targeted deletion of MC4-R in mice (Huszar *et al.* 1997), or rare MC4-R mutations in the human (Lubrano-Berthelier *et al.* 2006) result in an obese phenotype and hyperphagia. MC3-R knockout mice are also obese, but unlike MC4-R knockouts, these mice are not hyperphagic (Chen *et al.* 2000). Thus a reduction in energy expenditure is likely to be evident in these MC3-R knockout animals. Additionally, post-mortem studies as well as *in vitro* and *in vivo* work in humans have implicated β-MSH as a key mediator of body weight in humans (Biebermann *et al.* 2006).

Food restriction significantly increases the density of MC4-R in the hypothalamus of rats, whereas diet-induced obesity causes MC4-R expression to decrease, with MC3-R displaying no significant change in either model (Harrold *et al.* 1999). In contrast, MC3-R and MC4-R mRNA expression and/or distribution in the hypothalamus of the sheep does not change with long-term alterations in metabolic status and body weight (Iqbal *et al.* 2001a). Furthermore, POMC mRNA levels are reduced in lean fasted rats (Korner *et al.* 2001) and in sheep subjected to chronic food restriction (McShane *et al.* 1993) and leptin treatment is able to reverse these fasting induced changes in POMC gene expression in the rat (Korner *et al.* 2001). Others, however, have not been able to replicate this effect of altered body weight on POMC expression and may be due to variation between animals (Henry *et al.* 2000). Leptin has additionally been found to rapidly rewire the

synaptic input to POMC neurons resulting in an increase of POMC tone (Pinto *et al.* 2004).

1.9.5.4 Melanocortins and reproduction

It is generally accepted that melanocortins stimulate the reproductive axis. Treatment of humans with α-MSH (Limone *et al.* 1997) and rats with γ-MSH (Stanley *et al.* 2003) stimulates LH release. It has also been shown that γ-MSH causes a release of GnRH from rat hypothalamic GT-1 cell lines *in vitro* (Stanley *et al.* 2003). Additionally, α-MSH induces ovulation in female rats pre-treated with progesterone (Alde and Celis 1980) and treatment with the melanocortin endogenous inverse agonist, AGRP, prevents the steroid-induced LH and prolactin surges in the female rat (Schioth *et al.* 2001). Furthermore, MC4-R knockout mice have lowered fertility (Tucker *et al.* 2008). Conversely, melanocortin agonist, MTII, has no effect on gonadotropin levels in male *ob/ob* mice (Hohmann *et al.* 2000).

Melanocortins are thought to modulate the reproductive axis in times of negative energy balance by receiving signals from leptin. Treatment with the MC3-R/MC4-R antagonist SHU9119, or a selective MC4-R antagonist HS014, is capable of reducing the effectiveness of leptin to restore the LH surge in starved rats (Watanobe *et al.* 1999a). This study also suggests that the MC4-R is the principal melanocortin receptor involved in stimulating the reproductive axis. Others reject this notion after treatment with SHU9119 attenuated leptin's effects on food intake and body weight, but did not elicit any change to leptin's stimulatory effect on reproduction in the leptin treated *ob/ob* male mouse (Hohmann *et al.* 2000).

Converging lines of evidence support a stimulatory role for the melanocortins on reproductive function, but whether melanocortins exert a direct effect on GnRH neurons remains unclear. Melanocortin receptors are located in the POA (Iqbal *et al.* 2001a) where GnRH neurons are located, but whether these receptors are actually expressed on GnRH neurons remains to be ascertained. Earlier studies from our laboratory discovered a lack of direct input to GnRH neuronal cell bodies from the ARC (Pompolo *et al.* 2001), but there were projections from ARC to POA, close to GnRH cells, suggesting an intermediary neuron in the POA. In the rat, others have shown that β-END immunoreactive boutons do indeed contact GnRH

cells (Chen *et al.* 1989). Indeed, it is possible that there is axon-dendrite input to GnRH neurons that is not seen with conventional anterograde tracing studies or measurement of close appositions of the axonal elements and the perikaryon. In order to determine if this is the case, it may be warranted to conduct studies in animals treated with colchicine, which would highlight dendrites in the target cells.

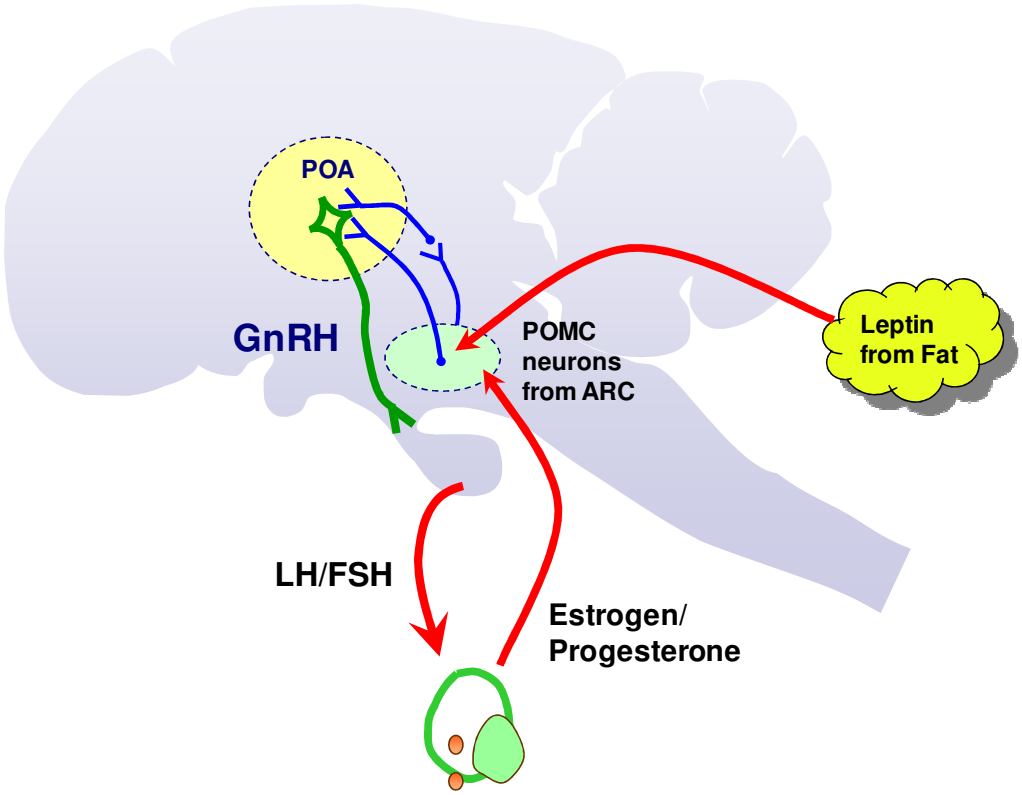

Figure 1.4: Leptin is secreted by fat in response to adiposity and acts on POMC cells in the arcuate nucleus to modulate GnRH secretion. This effect may be direct or may occur via interneurons. Altered GnRH secretion leads to altered secretion of LH and FSH, and therefore reproductive capabilities are modified. Sex steroids, estrogen and progesterone also modulate POMC neurons either directly or indirectly, again altering GnRH and subsequently LH and FSH secretions.

1.9.6 Agouti regulated peptide (AGRP)

There are two endogenous factors that act to block melanocortin action, namely, agouti and AGRP, which act selectively at the level of the melanocortin receptors. AGRP is co-expressed with NPY in ARC cells (Hahn *et al.* 1998) and acts only on CNS receptors, restraining the POMC pathway as an inverse agonist at the MC3-R and MC4-R (Nijenhuis *et al.* 2001). AGRP is equipotent at MC3-R and MC4-R and its effects on appetite and reproduction are the opposite to the melanocortins, thereby increasing food intake and body weight (Ebihara *et al.* 1999) and inhibiting reproductive function in rodents (Schioth *et al.* 2001; Vulliemoz *et al.* 2005).

Extensive research implicates AGRP as a negative regulator of leptin action. AGRP is up regulated in *ob/ob* mice and fasted rodents, and leptin treatment inhibits AGRP expression (Ebihara *et al.* 1999; Mizuno and Mobbs 1999; Korner *et al.* 2001; Duan *et al.* 2007). Chronic food-restriction in sheep has also been reported to result in an increase of AGRP mRNA expression (Henry *et al.* 2001b; Adam *et al.* 2002; Archer *et al.* 2004), which is expressed at low levels in normally fed animals (Henry *et al.* 2001b).

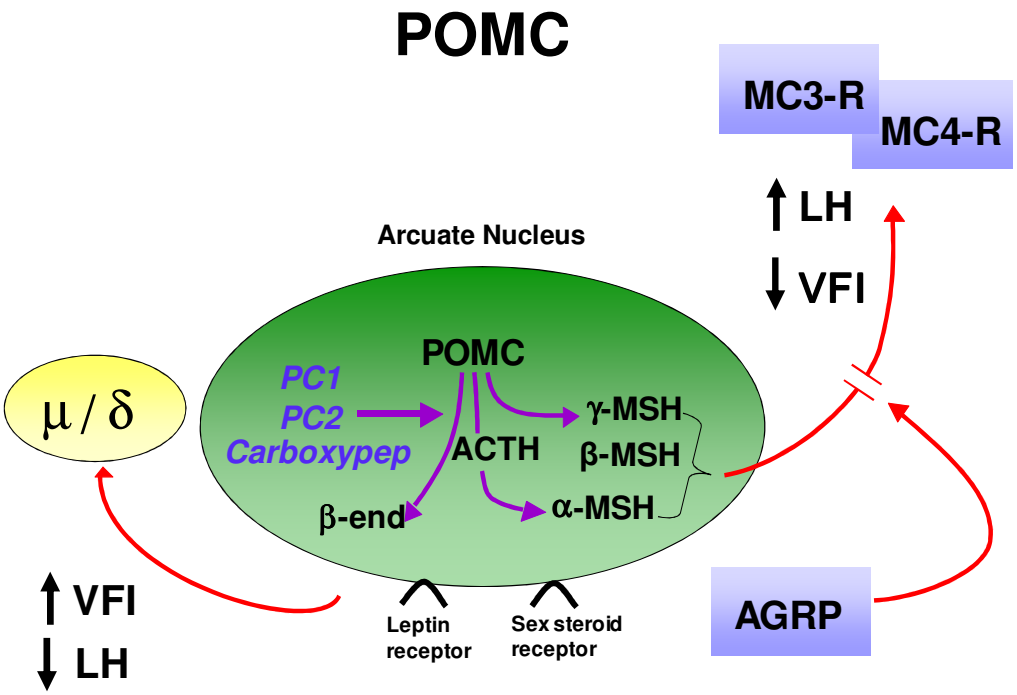

Figure 1.5: POMC containing neurons are located exclusively in the ARC of the ewe and are transcribed to produce the melanocortins which act via the MC3-R and MC4-R in the brain to stimulate reproduction and inhibit food intake. POMC is also transcribed to the endogenous opioid, β-END, which has opposing effects to melanocortins, increasing food intake and inhibiting LH secretion via the μ and δ receptors. AGRP acts as an endogenous inverse agonist to the melanocortin system antagonising the effects of the melanocortins at the MC3-R and MC4-R. POMC cells express both leptin and estrogen receptors and may be involved in mediating the effects of body energy stores and sex steroids on the reproductive system.

1.10 Kisspeptin

The Kiss1 gene was originally isolated in the ganglia of the venus clam on the basis of its cardiovascular properties (Price and Greenberg 1977). Further advancements in 1996 discovered anti-metastatic properties of this gene and the name 'metastin' was established (Lee *et al.* 1996b), until in 2001 kisspeptin was discovered as the natural ligand for the previously orphaned G-coupled protein receptor GPR54 (Kotani *et al.* 2001). The Kiss1 gene encodes a precursor protein of 145 residues that is processed to shorter forms sharing a common Arg-Phe C-terminal, which is highly conserved between the human and mouse (Stafford *et al.* 2002). In humans, the kisspeptin precursor is cleaved by an unknown mechanism into a 54 amino acid protein (Ohtaki *et al.* 2001) and then to three shorter products, being kisspeptin-14, -13, and -10 (Kotani *et al.* 2001).

1.10.1 Central distribution of kisspeptin and its receptor GPR54

Kiss1 mRNA is most abundantly expressed in the trophoblast tissue of the placenta, and in the CNS (Muir *et al.* 2001). In the rodent brain, *in situ* hybridisation and immunohistochemical analysis has revealed Kiss1 localisation in the anterodorsal preoptic nucleus, amygdala, the nucleus of the stria terminalis (BnST), brain stem and spinal cord, but the most abundant expression is located in the anteroventral periventricular nucleus (AVPV) and the ARC (Brailoiu *et al.* 2005; Smith *et al.* 2005a). In the ovine species a high proportion of kisspeptin immunoreactive cells (Franceschini *et al.* 2006; Pompolo *et al.* 2006) and Kiss1 mRNA (Estrada *et al.* 2006) is observed in the medial POA and the ARC. Smaller populations of kisspeptin immunoreactive cells are found in the periventricular nucleus of the hypothalamus and in scattered quantities outside of the hypothalamus extending from the diagonal band of Brocca through to the caudal POA (Pompolo *et al.* 2006), but these may be cells that produce a cross-reacting species because no Kiss1 mRNA expression is seen in these same areas (JT Smith, personal communication). Kisspeptin immunoreactive fibres are ubiquitously spread throughout the CNS with high densities found in the ARC and POA in both the rat (Brailoiu *et al.* 2005), mouse (Clarkson and Herbison 2006) and sheep (Franceschini *et al.* 2006). Additionally, a dense network of kisspeptin immunoreactive fibres was observed

within the external, neurosecretory zone of the median eminence of the sheep (Pompolo *et al.* 2006) but not in the mouse (Clarkson and Herbison 2006). It is important to note that different antisera to kisspeptin have produced differing results, and kisspeptin immunohistochemistry should be viewed with some circumspection. Immunohistochemistry studies first reported co-localisation of kisspeptin in GnRH neurons (Pompolo *et al.* 2006), yet others did not substantiate this finding after using a different antiserum (Franceschini *et al.* 2006). It is clear, therefore, that some antisera identify cross-reacting RF-amide species. The kisspeptin antiserum produced by Dr Caraty (Franceschini *et al.* 2006) is the only one available at present which has been successfully tested for cross-reactivity with other RF amides, and appears to be highly specific to ovine kisspeptin (Goodman *et al.* 2007).

GPR54 expression has also been detected in the human placenta and pancreas using semi-quantitative PCR (Muir *et al.* 2001; Ohtaki *et al.* 2001). In the rat brain, *in situ* hybridisation revealed GPR54 expression in the pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, and striatum (Lee *et al.* 1999). Specifically within the rat hypothalamus, GPR54 mRNA expression is found in the zona incerta, ARC and the DMH (Lee *et al.* 1999). Moreover, close examination by double-label *in situ* hybridisation revealed that virtually all GnRH neurons co-express GPR54 in the rat (Irwig *et al.* 2004), mouse (Han *et al.* 2005) and sheep (Smith, Unpublished data). Accordingly, kisspeptin fibres make close appositions with GnRH neurons in rats (Kinoshita *et al.* 2005), mice (Clarkson *et al.* 2009) and sheep (Smith *et al.* 2008a), but it is likely that these GnRH connections arise from kisspeptin neurons residing within the POA, as very few ARC neurons project to the POA and make contacts with GnRH neurons in the ewe (Pompolo *et al.* 2001). It is also probable that kisspeptin cells of the ARC make indirect connections with GnRH neurons via a series of interneurons. Preliminary data from our laboratory show kisspeptin immunoreactive fibres in close apposition to various other cell types in the ovine brain including cells that produce the major excitatory neurotransmitter, glutamate (van den Pol *et al.* 1990), as well as cells that produce the inhibitory neurotransmitters, gamma-Aminobutyric acid (GABA) and dopamine (Smith et al, Unpublished data). Glutamate is implicated in the direct regulation of GnRH, and

glutamate-GnRH contacts increase at the time of the LH surge in the rat (Ottem *et al.* 2004). GABA amino acid release, on the other hand, is reduced at the time of the LH surge (Jarry *et al.* 1995) and GABA treatment in the POA is able to block the LH surge in rats (Herbison and Dyer 1991). Dopaminergic neurons inhibit GnRH secretion in the rat (Meyer and Goodman 1985). Kisspeptin cells are therefore well positioned within the brain to act directly and indirectly to modulate GnRH secretion, however further work is needed to decipher such inter-neuronal pathways.

1.10.2 Kisspeptin regulation of reproduction

The reproductive importance of the kisspeptin/GPR54 system was revealed in 2003 when results from two independent groups discovered that disabling mutations of GPR54 resulted in hypogonadotropic hypogonadism (de Roux *et al.* 2003; Seminara *et al.* 2003). Advances in kisspeptin reproductive research since this discovery has been rapid, and unequivocal evidence substantiates the compelling ability of the kisspeptin system to stimulate the reproductive axis in the rodent (Gottsch *et al.* 2004; Irwig *et al.* 2004), sheep (Smith *et al.* 2006b), monkey (Shahab *et al.* 2005), and human (Dhillo *et al.* 2005). The kisspeptin products and GPR54 are recognised as essential gatekeepers of puberty onset, whereby an increase in kisspeptin tone and sensitivity is considered to be vital for the initiation of puberty (Navarro *et al.* 2004b; Shahab *et al.* 2005; Roa *et al.* 2006).

Kisspeptin cells are well positioned to act as a conduit for sex steroid feedback action on GnRH secretion, and may act either directly or indirectly on GnRH neurons to modulate its secretion. Kisspeptin cells project to GnRH neurons (Clarkson and Herbison 2006), which all express GPR54 mRNA (Irwig *et al.* 2004), and virtually all ARC kisspeptin neurons (Franceschini *et al.* 2006; Smith *et al.* 2007) and approximately 50% of POA kisspeptin (Franceschini *et al.* 2006) neurons express sex steroid receptors. Furthermore, sex steroids regulate Kiss1 expression (Smith *et al.* 2005b; Smith *et al.* 2007) (See section 1.10.3 for more detail of sex steroid control of kisspeptin). Moreover, kisspeptin administration to the POA, where most GnRH neurons are located, increases plasma LH secretion, and blockade of kisspeptin action within the POA completely abolishes the pro-estrous

LH surge and inhibits estrous cyclicity (Kinoshita *et al.* 2005). Central administration of kisspeptin to sheep increases plasma LH concentrations and produces a substantial release of GnRH into the cerebral spinal fluid (Messager *et al.* 2005). Lastly, central treatment with a kisspeptin antagonist is able to inhibit firing of GnRH neurons in the mouse and reduce pulsatile GnRH secretion in female pubertal monkeys (Roseweir *et al.* 2009). This study further revealed that kisspeptin antagonist treatment to sheep, mice or rats blocks the increase in LH secretion typically seen following gonadectomy, suggesting that kisspeptin neurons are capable of mediating negative feedback effects of sex steroids to the pituitary gonadotropes (Roseweir *et al.* 2009).

There has been some debate as to whether kisspeptin exerts its effects at the level of the hypothalamus or pituitary. Studies in our group support a hypothalamic target after kisspeptin treatment to hypothalamo-pituitary-disconnected ewes was unable to elicit an LH response (Smith *et al.* 2008b), however several studies suggest a pituitary action after kisspeptin, albeit at supraphysiological concentrations, increased LH secretion from pituitary cell cultures *in vitro* (Navarro *et al.* 2005; Smith *et al.* 2008b).

1.10.3 Regulation of the Kiss1 gene by sex steroids

Kiss1 mRNA expression is under the control of sex steroids in both the rodent and ovine species, and kisspeptin cells are believed to regulate both the negative and positive feedback effects of estrogen on GnRH neurons in a site specific manner. Hypothalamic Kiss1 mRNA expression in male and female rats is increased following gonadectomy and is normalised following sex steroid replacement (Navarro *et al.* 2004a), an effect that is prominent in the ARC of females (Irwig *et al.* 2004). The opposite is observed in the AVPV of the mouse brain whereby Kiss1 gene expression is reduced following gonadectomy and is increased with sex steroid treatment (Smith *et al.* 2005a; Smith *et al.* 2005b). Others have shown that Kiss1 mRNA is dramatically up regulated in the AVPV of the rat on the evening of proestrus, and kisspeptin neurons in this nucleus co-express the immediate early gene fos at the time of the GnRH/LH surge, whereas kisspeptin neurons in the ARC are fos-negative during this surge phase (Smith *et al.* 2006c). Importantly, previous

research shows that in rodents the GnRH/LH surge is generated by estrogen sensitive neurons located in the AVPV (Gu and Simerly 1997). Collectively, these studies indicate that kisspeptin neurons located in the AVPV are involved in the *positive* sex steroid feedback regulation of GnRH neurons to generate the pre-ovulatory LH surge, while those kisspeptin neurons in the ARC are involved in the sex steroid *negative* feedback actions (Figure 1.6).

Estrogen also negatively regulates Kiss1 mRNA expression (Smith *et al.* 2007) and peptide (measured by immunohistochemistry) (Smith *et al.* 2008a) in the ARC of the sheep, and ovariectomy of the sheep increases the number of immunohistochemically identified kisspeptin cells in this nucleus (Pompolo *et al.* 2006). The Kiss1 gene is also involved with the positive feedback of estrogen and its gene expression is increased in the caudal ARC immediately prior to the GnRH/LH surge (Estrada *et al.* 2006), representative of an acute response to the rise in estrogen levels (Figure 1.6). Consistent with this, is the finding that the positive feedback effects of estrogen on GnRH secretion are promulgated through the mediobasal hypothalamus (including the ARC) in the ewe (Blache *et al.* 1991). It is unknown whether sub-populations of kisspeptin neurons within the ARC are differentially regulated by estrogen, or if the ARC kisspeptin cells respond differently, dependent upon different conditions (acute vs chronic effects of estrogen).

In contrast to the rat, the sheep does not express a population of kisspeptin neurons in the AVPV, but there is a population of kisspeptin neurons observed in the medial POA (Franceschini *et al.* 2006; Pompolo *et al.* 2006). The functional significance of the kisspeptin cells in the POA of the sheep remains to be fully elucidated, however it has been proposed that these cells may act in a similar manner to the kisspeptin cells in the AVPV of the rodent, to positively regulate GnRH/LH secretion during the pre-ovulatory LH surge (Smith 2008) .

1.10.4 Kisspeptin/GPR54 and the metabolic control of reproduction

The kisspeptin system has only recently emerged as a possible mediator of systemic metabolic signals to central reproductive control, nonetheless, consistent results in rats and mice demonstrate that negative energy balance results in reduced Kiss1 gene expression (Castellano *et al.* 2005; Smith *et al.* 2006a; Luque *et al.* 2007). Moreover, treating pre-pubertal undernourished rats with kisspeptin-10 restores pubertal activation of the reproductive axis (Castellano *et al.* 2005). Finally, Kiss1 mRNA expression is decreased in NPY knockout mice and NPY stimulates Kiss1 gene expression in hypothalamic N6 cell lines (Luque *et al.* 2007).

1.10.5 Kisspeptin and leptin

Research has implicated the satiety hormone leptin as a metabolic regulator of the hypothalamic kisspeptin system. Kisspeptin cells in the ARC of the mouse express leptin receptors (Smith *et al.* 2006a), and leptin treatment increases Kiss1 mRNA when administered to the N6 murine hypothalamic mouse cell line (Luque *et al.* 2007). Moreover, ARC Kiss1 mRNA is decreased in leptin deficient *ob/ob* mice, but expression is increased with leptin treatment (Smith *et al.* 2006a; Luque *et al.* 2007). Consequently, it is tempting to speculate that the kisspeptin system may also regulate food intake and body weight homeostasis, but mice and humans with disruptions to GPR54 display no major perturbation of food intake or body weight regulation (de Roux *et al.* 2003; Seminara *et al.* 2003). In these models there may be some compensatory mechanism to maintain homeostatic balance. Acute kisspeptin treatment to lean animals at a dose that stimulates LH secretion does not alter food intake (Thompson *et al.* 2004; Castellano *et al.* 2006). It would be interesting to administer kisspeptin by prolonged infusion to clearly determine any effects on food intake.

Collectively, the available data are suggestive of leptin regulation of kisspeptin neurons in the ARC of rodent species. The question as to whether other populations of kisspeptin neurons, such as those in the AVPV in the rat or POA in the sheep, also play a role in the metabolic regulation of reproduction remains to be examined.

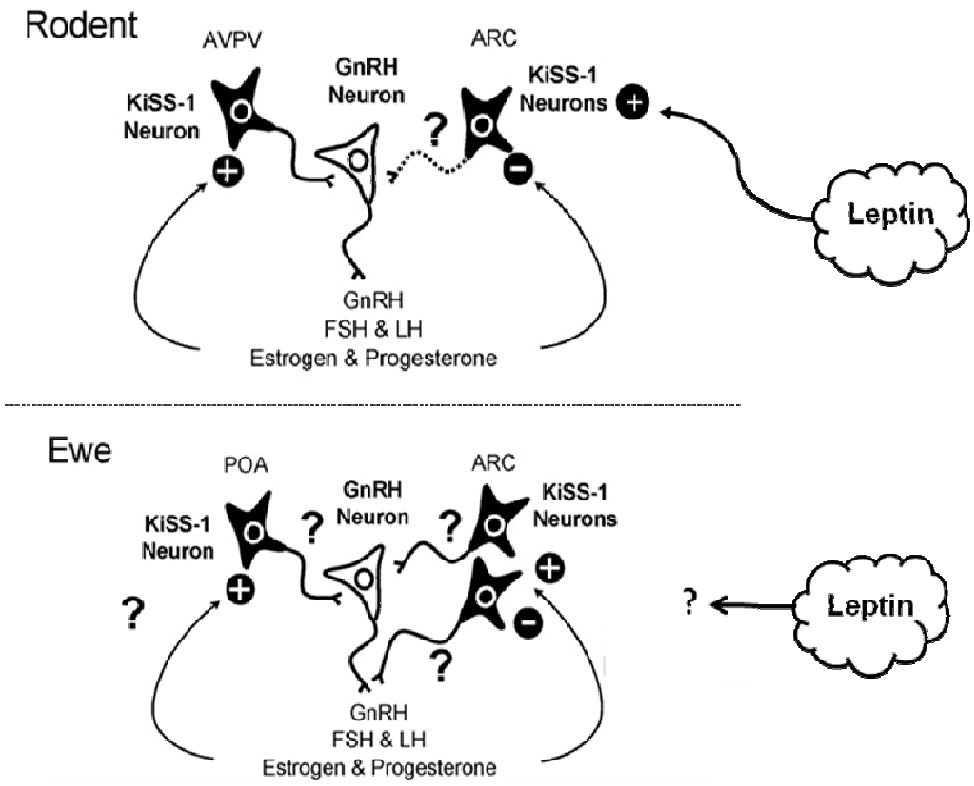

Figure 1.6: Proposed sex steroid and leptin feedback to kisspeptin cells in the rodent and the ewe. In the rodent, estrogen and progesterone stimulates the expression of Kiss1 in the AVPV and thus stimulates GnRH, mediating positive feedback regulation. Conversely, sex steroids inhibit Kiss1 expression in the ARC, reducing GnRH and exerting negative feedback regulation. In the ewe sex steroids both inhibit and stimulate Kiss1 mRNA expression in the ARC, and is proposed to be involved in both negative and positive feedback regulation of GnRH neurons. Kisspeptin neurons in the POA of the ewe are up regulated by sex steroids, however the functional significance of this population of kisspeptin neurons remains to be determined. Leptin stimulates Kiss1 mRNA in the rodent, but the effect of leptin on kisspeptin in the ewe has not yet been revealed. Modified from (Smith 2008).

1.11 Orexin (ORX)

Orexins, also known as hypocretins, are a novel family of peptides which were characterised in 1998 (de Lecea *et al.* 1998; Sakurai *et al.* 1998). The name 'orexin', a Greek word which means appetite, was derived from the original observation that the peptides play a role in regulating food intake (Sakurai *et al.* 1998). Orexin A and B (ORX-A and ORX-B) (or hypocretins A and B), are produced from a common precursor polypeptide, prepro-orexin (ppORX)(Sakurai *et al.* 1998) and are concerned with the regulation of energy metabolism (Sartin *et al.* 2001), neuroendocrine reproductive function (Small *et al.* 2003) and sleep and arousal states (Lee *et al.* 2005). ORX actions are mediated via Orexin 1 (OX-R1) and Orexin 2 (OX-R2) receptors and mRNA for this precursor is detected exclusively in the brain of the rat (de Lecea *et al.* 1998). ORX-A is conserved across rat, bovine and human, with an identical structure, whereas rat ORX-B shares 95% homology with the mouse and only 85% with the human (Sakurai *et al.* 1998).

1.11.1 Central distribution of Orexin and its receptors

The distribution of ORX neurons were first characterised in the rat and mouse using *in situ* hybridisation by Sakurai *et al.* (1998) and Broberger *et al.* (1998); both groups reporting the existence of the cells in the LHA and the PFA. Others further confirmed this result in rats (Cutler *et al.* 1999) and monkeys (Horvath *et al.* 1999) using immunohistochemical studies. In the rat, ORX-A and ORX-B has also been located in the pituitary gland with higher concentrations of both types in the posterior lobe compared to the anterior lobe (Date *et al.* 2000). ORX-A immunoreactive fibres are densely distributed in the hypothalamus, septum, thalamus, locus coeruleus, spinal cord, and near the ventricles (Cutler *et al.* 1999), and more specifically, in the DMH, PVN, LHA, and most abundantly in the ARC (Horvath *et al.* 1999). Immunoreactive ORX fibres have also been reported in the BnST, anterior hypothalamus, medial POA and supra-optic nucleus (SON) of the rat (Peyron *et al.* 1998). The sheep shows a similar expression of ORX immunoreactive fibres and cells, but also has a population of ORX cells in the zona incerta (ZI), and the most abundant population is in the DMH with a few scattered cells in the ventrolateral part of the LHA (Iqbal *et al.* 2001c). Recent retrograde tracing studies

in the ewe have reported ORX projections to the VMN and ARC (Qi *et al.* 2008) as well as to the PVN and the LHA (Qi *et al.* 2009a).

The distribution of ORX receptors has been extensively studied, with OX-R1 and OX-R2 exhibiting distinct distributions patterns within the rodent brain. ORX-1 mRNA is most abundantly observed in the VMN whereas OX-R2 is predominant in the PVN (P. Trivedi et al., 1998; X. Y. Lu et al., 2000). Further gene expression for OX-R1 has been located in the DMH, POA, VMH, cerebral cortex, posterior and anterior pituitary, basal ganglia, hippocampus, dorsal raphe nucleus and the locus coeruleus (Trivedi *et al.* 1998; Date *et al.* 2000; Lu *et al.* 2000; Hervieu *et al.* 2001; Marcus *et al.* 2001). OX-R2 mRNA has been further observed in the DMH, ventral premammillary nucleus, nucleus accumbens, cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, and raphe nuclei (Trivedi *et al.* 1998; Lu *et al.* 2000; Marcus *et al.* 2001). The location of ORX receptors in the hypothalamus is consistent with its role in modulating food intake and reproduction.

1.11.2 Orexin and food intake

ORX-A (Sakurai *et al.* 1998) and ORX-B (Sartin *et al.* 2001) stimulates food intake, genetic ablation of ORX neurons in mice results in obesity (Hara *et al.* 2001; Hara *et al.* 2005), and icv administration of anti-ORX antibody or an OX-R1 selective antagonist reduces food intake (Haynes *et al.* 2000; Yamada *et al.* 2000). Moreover, antagonising the OX-R1 in leptin deficient *ob/ob* mice reduces food intake and ameliorates obesity (Haynes *et al.* 2002), suggesting that leptin deficiency at least partly activates the ORX pathway to increase food intake. Consistent with this, 100% of ovine ORX cells express Ob-Rb (Iqbal *et al.* 2001b), and ORX mRNA is increased in the fasted state of rats (Sakurai *et al.* 1998). Expression of the ORX gene does not change in long-term food–restricted, lean sheep (Iqbal *et al.* 2003), so the role of the peptide in this species with respect to food intake is not certain.

1.11.3 Orexin, reproduction and sex steroids

Knowledge of the involvement of the ORX system in the regulation of GnRH/LH secretion is somewhat confusing with studies reporting both stimulatory and inhibitory actions depending on steroid milieu. ORX-A stimulates LH secretion in steroid-primed OVX rats, but suppresses LH secretion in OVX animals not primed with steroids (Pu *et al.* 1998). Consistent with this notion, treatment with ORX-A is able to rescue the steroid-induced LH surge in 3-day fasted rats (Kohsaka *et al.* 2001). Conversely, icv administration of ORX-A reduces the mean concentration and pulse frequency of LH secretion in non-steroid primed OVX rats (Irahara *et al.* 2001).

It is suggested that ORX may exert a site-specific effect on the reproductive axis, stimulating LH secretion when administered to the rostral POA, and inhibiting LH secretion following injection to the medial POA or ARC/median eminence (Small *et al.* 2003). In accordance with a reproductive role for ORX neurons, 85% of GnRH neurons in the rat express OX-R1 and receive direct contact from ORX fibres (Campbell *et al.* 2003; Small *et al.* 2003). Likewise, in the sheep, one third of GnRH cells in the ovine POA display close contact with ORX varicosities, strongly suggesting a direct input (Iqbal *et al.* 2001c). In spite of the accumulating data implicating ORX in the modulation of reproductive function, ORX does not seem to be fundamental to reproductive success as ORX knockout mice are fertile (Chemelli *et al.* 1999).

ORX neurons do not appear to be regulated by sex steroids in the female rat as ovariectomy and further estrogen treatment has no effect on ORX gene expression (Russell *et al.* 2001), and Immunolabeling for ORX and ER-α reveals no co-localisation (Muschamp *et al.* 2007). Others however, have shown that ORX-A mRNA is up regulated in the pro-estrous phase of the rat estrous cycle (Porkka-Heiskanen *et al.* 2004), and, *in vitro* studies have demonstrated estrogen up regulation of ORX mRNA from hypothalamic explants in the male rat (Russell *et al.* 2001). Furthermore, ovariectomy has been shown to up regulate OX-R1 in the anterior pituitary of the rat, and estrogen replacement inhibits this increase (Johren *et al.* 2003), suggesting estrogen regulation of the ORX receptor.

1.12 Neuropeptide Y

Neuropeptide Y, a 36 amino acid peptide, was first isolated from porcine brain in 1982 by Tatemoto (Tatemoto 1982). Since its discovery, it has been isolated in many other species, including the human, rat, and sheep. Computer assisted analysis has revealed that this peptide consists of two main domains, the C-terminal region and the N-terminal portion (Danger *et al.* 1990). These features have classified NPY into the PP family, with structural similarities to both peptide tyrosine-tyrosine (PYY) and pancreatic polypeptide (PP).

NPY elicits a plethora of physiological actions. It has important effects on vasoconstrictor behaviour, cerebral blood flow, circadian rhythms, melatonin biosynthesis, and cardiac and respiratory frequencies (Danger *et al.* 1990). Widely studied effects of NPY indicate involvement of NPY in reproduction and appetite. NPY increases food intake (Clarke *et al.* 2005) and has been found to be an important modulator of the hypothalamic-pituitary-gonadal axis, controlling the release of GnRH and LH in sheep (Barker-Gibb and Clarke 1996) as in other species (Kalra and Kalra 2004).

1.12.1 Central distribution of NPY and receptors

NPY is the most abundant peptide found in the mammalian central and peripheral nervous system. Mapping of NPY neurons in the brain of both the rat, by indirect immunofluorescence (Chronwall *et al.* 1985), and the sheep, by immunohistochemical double-labelling (Tillet *et al.* 1989) has demonstrated that NPY innervation of the hypothalamus originates from two major sites. The first and major site of origin is the ARC of the hypothalamus (Bai *et al.* 1985), which is also the main site of NPY synthesis (Morris 1989). These cells then project to the median eminence, PVN (Kalra *et al.* 1987) and POA (Li *et al.* 1999). The second site of origin of NPY is the brain stem, where these cells are co-localised in noradrenergic cells of the A1 area (Tillet *et al.* 1993), and project to innervate the median eminence, medial POA and other hypothalamic nuclei (Tillet *et al.* 1989; Kalra 1993).

NPY containing terminals in the POA and median eminence come in close proximity with GnRH neurons in both the rat (Tsuro 1990) and sheep (Norgren and Lehman 1989; Tillet *et al.* 1989), with one study detecting synaptic contacts between GnRH cell bodies and NPY terminals in the POA of the rat brain (Smith and Jennes 2001). Furthermore, double immunostaining revealed that NPY endings in the POA have a similar distribution pattern as GnRH in the rat (Guy *et al.* 1988). These studies are all suggestive of neuronal communication between NPY and GnRH whereby NPY may regulate GnRH and thus LH secretion.

In contrast to these findings, anterograde tracing studies in the sheep strongly suggests that there are no direct inputs from the ARC to GnRH immunoreactive cells in the sheep (Pompolo *et al.* 2001), so input to GnRH cells may be via at least one interneuron.

To date, six receptor sub types (Y1-Y6) have been found to mediate the actions of NPY based on their affinities for NPY receptor agonists, with each receptor able to elicit different physiological responses. These receptors have been studied in some detail in the sheep, with the exception of the Y3 and Y6. The Y3 receptor has not yet been cloned (Balasubramaniam 2002), and Y6 appears to be completely missing from the rat genome, and exists in a truncated non-functional form in primates (Parker and Herzog 1999). We have previously shown in the ewe that stimulating the Y1 receptor stimulates food intake, whereas a Y2 receptor antagonist is able to delay the estrogen-induced LH surge (Clarke *et al.* 2005).

1.12.2 NPY and food intake

NPY unabatedly stimulates food intake and encourages weight gain in rats (Stanley *et al.* 1986), and sheep (Miner *et al.* 1989). Chronic central administration of NPY produces an obesity syndrome characterised by increased adiposity, insulin resistance, hypercorticism, and hypogonadism, reminiscent of the phenotype seen in the *ob/ob* mouse, that displays elevated hypothalamic NPY secondary to lack of leptin negative feedback action (Raposinho *et al.* 2001). NPY containing neurons in the ARC have been shown to express leptin receptors in rats (Stephens *et al.* 1995) and sheep (Iqbal *et al.* 2001b), and central administration of leptin consistently

down-regulates NPY mRNA expression in the ARC of fed and fasted rodents (Wang *et al.* 1997; Sahu 1998; Ahima *et al.* 1999) and in *ad lib* fed sheep (Henry *et al.* 1999). Obesity and hyperphagia in the *ob/ob* mouse is attenuated by NPY gene deletion (Erickson *et al.* 1996).

1.12.3 Effects of NPY on LH secretion

NPY has clearly been shown to play a critical role in the modulation of LH secretion in several mammalian species, however the precise actions of NPY varies between species and endocrine states. NPY both inhibits and stimulates LH release depending on steroid milieu in the rat. Many studies have shown *suppression* of episodic release of LH after intracerebral administration of NPY to sex steroid deficient, OVX rats (Kalra and Crowley 1984; Sabatino *et al.* 1989). However, when intact, or primed with steroids during NPY infusion, rats display an *increase* in LH secretion (Kalra and Crowley 1984). This result is similar in other animals including the rabbit (Kaynard *et al.* 1990) and monkey (Woller and Terasawa 1994).

This inhibitory effect is also seen in OVX sheep (Malvern *et al.* 1992; Clarke *et al.* 2005), and cows (Thomas *et al.* 1999), but when treated with steroids, they do not exhibit the stimulatory actions seen in rats (McShane *et al.* 1992). Rather, when treated with ovarian steroids, inhibitory effects of the peptide are still seen (McShane *et al.* 1992), or show no change (Porter *et al.* 1993). In the sheep therefore, exogenous NPY has only inhibitory actions on GnRH/LH secretion.

The role that NPY has to play in the regulation of the LH surge has also been studied in some depth, albeit with some contradictory results. Central administration of NPY (Estrada *et al.* 2003a) or a NPY Y2 selective agonist (Clarke *et al.* 2005) in the third ventricle of sheep caused a delay or completely blocked the LH surge. This inhibitory effect of NPY in sheep has further been revealed when central administration of NPY anti serum against a conjugate of porcine NPY with bovine thyroglobulin did not delay or block the LH surge in OVX, estrogen treated sheep (Malvern *et al.* 1995). In contrast, the LH surge of NPY knockout mice was diminished by 50% compared to their wild type counterparts, demonstrating that preovulatory NPY release is essential for normal amplification of the LH surge in

pro-estrous mice (Xu *et al.* 2000). Dynamic changes of hypothalamic NPY gene expression occurs in association with the LH surge, with a maximal NPY mRNA increase in the medial basal hypothalamus occurring just prior to the LH surge in ovarian steroid-primed OVX rats (Sahu *et al.* 1994). Bauer-Dantoin *et al* (1992) also demonstrated an increase in NPY mRNA in the ARC as LH concentrations increased across the estrous cycle in rats. In sheep, however, *in situ* hybridisation has revealed NPY mRNA expression in the ARC to be greatest during the luteal phase of the estrous cycle (Estrada *et al.* 2003a).

The increased NPY gene expression in the rat brain prior to the LH surge suggests that there may be a higher release of NPY which in turn increases the rate of LH release. However, in the sheep NPY mRNA expression is not increased during the preovulatory period, indicating that NPY may not play a role in the generation of the LH surge. The increase in mRNA in the luteal phase of the sheep also reinforces the notion that NPY plays an inhibitory role to LH secretion, as it is during this phase that progesterone is high and has a negative feedback effect on GnRH. It is likely that NPY mediates the effects of sex steroids via an interneuronal pathway as only a 10% of NPY neurons express sex steroid receptors (Skinner and Herbison 1997).

1.13 Regulation of NPY and POMC by Insulin and Glucose

Peripheral Insulin and glucose levels are implicated in the regulation of ARC appetite regulating peptides, NPY and POMC. Insulin is released into the peripheral circulation in proportion to adiposity and acts as a satiety factor (Woods *et al.* 1979). Neuron-specific deletion of insulin receptors leads to hypothalamic hypogonadism (Bruning *et al.* 2000), indicating that insulin sensing in the brain is essential for normal reproductive functioning. GnRH neurons do not express insulin receptors, but these receptors are present in the ARC (Baskin *et al.* 1987). Insulin regulates NPY gene expression, such that central administration of insulin to fasted mice results in decreased NPY mRNA expression in the ARC (Schwartz *et al.* 1991), and insulin deficient diabetic rats display an increase in NPY mRNA expression that is normalised by systemic insulin treatment (Abe *et al.* 1991). A high number of insulin receptors have also been noted on ARC POMC cells (Benoit

et al. 2002). Insulin action on POMC cells, however, is not required for metabolic homeostasis, as mice with targeted deletion of insulin receptors on POMC neurons do not display any alteration in metabolic profile (Konner *et al.* 2007).

Glucose concentrations are also decreased with negative energy balance and re-feeding restores plasma glucose levels back to normal which precedes the restoration of pulsatile LH secretion (Szymanski *et al.* 2007). In addition, inhibition of glucose oxidation suppresses pulsatile LH release in rats (Nagatani *et al.* 1996) and ewes (Bucholtz *et al.* 1996). POMC and NPY cells are two examples of glucose sensing neurons within the ARC. These neurons sense the changes in sugar levels within the peripheral circulation and increase or decrease firing depending on glucose levels. Patch-clamp recordings in mouse brain slices have recently illustrated excitory properties of glucose on POMC neurons (Ibrahim *et al.* 2003), however others, could not replicate this effect in the rat (Wang *et al.* 2004). Additionally, disrupted glucose sensing in mouse POMC neurons inhibits the glucose stimulated α-MSH release seen in wild type controls (Parton *et al.* 2007). Approximately 40% of ARC NPY neurons are inhibited by glucose in the rat (Mountjoy *et al.* 2007), and fasting increases glucose sensing to NPY neurons. Therefore, as a result of fasting reduced glucose concentrations, stimulation of POMC and inhibition on NPY neurons would be reduced, and may therefore enhance NPY neurotransmission.

1.14 Summary and Aims

Estrogen and leptin act in overlapping nuclei and neurons within the brain to regulate the reproductive and metabolic axis, however the mechanisms of action for these two factors remains an enigma. The melanocortins produced from the ARC POMC cells, are poised as key intermediaries in transmitting information regarding body fat stores and sex steroid feedback to areas in the brain involved in reproduction, possibly via an interneuronal pathway using other cells with dual appetite and reproductive functions as a conduit. Although there is a great deal of work expounding the role of the melanocortins stimulating the reproductive axis in rats that have been starved for 2-3 days, our sheep model is one which is more comparable to the human in which a hypogonadotropic state occurs due to a reduction in body reserves over a longer period of time. This is important because unlike the acute challenge presented to starved laboratory rats, our animals establish an altered metabolic set point to normal animals and are not compromised in terms of health. The general aim of this thesis was to seek further evidence that the melanocortin system is of fundamental importance to the normal reproductive process in the ewe. In particular, these studies aimed to provide significant new information on how 'dialogue' is achieved between energy stores and the reproductive system, as well as delineating the role of the POMC cells in the feedback of sex steroids on the GnRH and metabolic systems. This body of work aimed to illustrate the importance of the melanocortins in regulating metabolic information back to the reproductive cells in times when energy reserves are low. Ultimately, this work will add further impetus to the therapeutic intervention of restoring reproductive hormones in this negative energy state, thereby circumventing possible later life complications associated with reproductive suppression.

1.15 Hypothesis

The unifying hypothesis tested in this thesis was that the POMC derived melanocortins are fundamental stimulants of the reproductive axis. This family of peptides interact with other cells type within the brain to regulate GnRH secretion, and this is especially true in times of negative energy balance.

The specific hypotheses tested were;

1. That restoration of gonadotropic function is a result of an up regulation of central acetylated melanocortin production.
2. Melanocortins indirectly stimulate GnRH neurons.
3. Kisspeptin neurons respond to leptin status in a similar manner to melanocortins, and communicate with POMC and NPY cells to stimulate reproduction.

Chapter 2

Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes

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Declaration for Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiment management/conduct, laboratory analysis, data collection, statistical analysis, preparation of manuscript	60%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Iain J Clarke[1]	Provided intellectual input and editing of manuscript	N/A
Marissa Bowden[2]	Experiment conduct	10%
Kevin Gamber[3]	Intellectual Input/Experiment conduct	N/A
Christian Bjørbæk[3]	Provided intellectual input and editing of manuscript	N/A
Javed Iqbal[1]	Experiment conduct	N/A

Candidate's Signature		**Date**

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	[1]Monash University, Department of Physiology, Clayton Campus [2]Baker IDI Heart and Diabetes Institute, Melbourne [3]Beth Israel Deaconess Medical Centre and Harvard Medical School, Boston, Massachusetts

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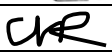
Signature 4		
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Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes

2.1 Abstract

Background/Aims: Leptin restores gonadotropic function in lean hypogonadotropic animals, by an unknown mechanism. We aimed to test the hypothesis that restoration of gonadotropic function is a result of an up regulation of central acetylated melanocortin production.

Methods and Results: Lean ovariectomised (OVX) ewes received intracerebroventricular (icv) infusions of leptin (or vehicle) for 3 days, which up regulated POMC mRNA and restored pulsatile luteinising hormone (LH) secretion. Melanocortin agonist (MTII), but not naloxone treatment, reinstated pulsatile LH secretion in lean OVX ewes. We treated (icv) lean OVX ewes with leptin (or vehicle) and measured peptide levels and post-translational modification in the ARC. Levels of β-END were lower in lean animals, with no effect of leptin treatment. Des-α-MSH was the predominant form of α-MSH in the ARC and levels were similar in all groups. In another group of lean and normal-weight OVX ewes, we measured the different forms of α-MSH in ARC, hypothalamus (ARC removed) and the preoptic area (POA). Acetylated α-MSH levels were lower in lean animals in the terminal beds of the hypothalamus and POA but not the ARC.

Conclusions: Leptin corrects the hypogonadotropic state in the lean condition by up regulation of POMC gene expression, and may increase transport and acetylation of melanocortins to target cells in the brain. Melanocortin treatment restores LH secretion in lean animals.

2.2 Introduction

Leptin is produced by adipose tissue and signals metabolic status to the brain to regulate food intake and energy expenditure (Friedman and Halaas 1998; Clarke *et al.* 2001). Leptin also restores reproductive function in animals genetically deficient in leptin (Mounzih *et al.* 1997) and restores the pulsatile secretion of luteinising hormone (LH) in lean ovariectomised (OVX) ewes that are hypogonadotropic (Henry *et al.* 2001a). Central action of leptin is transmitted via the signalling form of the leptin receptor (Ob-Rb), which is found in a variety of neurons, especially those that produce peptides/neurotransmitters that control food intake, metabolic function and energy expenditure (Iqbal *et al.* 2001b). Most of the so-called 'appetite-regulating peptides' also regulate reproductive function, acting within the brain (Clarke and Henry 1999) to regulate gonadotropin releasing hormone (GnRH) secretion (Crown *et al.* 2007). Neuropeptide Y (NPY), agouti related peptide (AGRP), and melanocortins are examples of peptides with such dual function (Nijenhuis *et al.* 2001; Schioth *et al.* 2001; Clarke *et al.* 2005; Cone 2005).

The proopiomelanocortin (POMC) cells of the arcuate nucleus (ARC) also express Ob-Rb and a proportion of these express estrogen receptor-α (ERα) in rats (Morrell *et al.* 1985; Cheung *et al.* 1997) and sheep (Lehman and Karsch 1993; Iqbal *et al.* 2001b). The POMC pro-hormone is post-translationally cleaved to produce β-endorphin (β-END) and the melanocortins, α-melanocyte stimulating hormone (α-MSH), β- and γ-MSH (Pritchard *et al.* 2002). Endogenous opioids, including β-END, negatively regulate reproduction (Bonavera *et al.* 1993) and stimulate food intake (Grandison and Guidotti 1977). On the other hand, the melanocortins reduce food intake (Fan *et al.* 1997) and stimulate reproduction (Watanobe *et al.* 1999a), acting via the melanocortin receptors (MC-R), MC-3R and MC-4R. The generation of α-MSH and β-END from the same POMC precursor involves complex post-translational processing by prohormone convertase 1 and 2 (PC1 and PC2). In addition, carboxypeptidase E (CPE) 'trims' the carboxy terminus and the peptidyl α-amidating mono-oxogenase enzyme (PAM) amidates the C terminus to enable bio-activity. Acetylation of the N-terminus increases the ability of α-MSH to reduce food intake, but acetylation of β-END nullifies activity (Tsujii and Bray 1989; Abbott

et al. 2000). The enzyme(s) (acetylase) responsible for this process have not been identified.

With respect to melanocortin action on the reproductive axis, Watanobe *et al* (1999a) showed that the MC3-R/MC4-R antagonist SHU9119 or HS014 (a selective MC4 receptor antagonist) could reduce the effectiveness of leptin to restore gonadotropin secretion in starved rats. Other work indicated that α-MSH (Limone *et al.* 1997) and γ-MSH (Stanley *et al.* 2003) are sufficient to stimulate LH release in humans and γ-MSH causes GnRH release from hypothalamic explants (Stanley *et al.* 2003). On the other hand, the MC3-R/MC4-R agonist melanotan-II (MTII) had no effect on gonadotropin levels in male *ob/ob* mice (Hohmann *et al.* 2000), suggesting a possible species difference.

An ovine model of long-term restricted feeding results in a hypogonadotropic condition which, unlike the starved rat, is not catabolic, and is more comparable to the human condition of lean condition caused by dieting or over-exercise. In contrast the to rat where POMC cells are located in ARC and the NTS (Guo *et al.* 2004), POMC cells are located exclusively in the ARC in both sheep (Clarke, unpublished data) and humans (Zaphiropoulos *et al.* 1991) and both of these species are able to produce β-MSH (Seidah *et al.* 1979; Bertagna *et al.* 1989), which is not produced from the rodent POMC precursor (Pritchard and White 2007).

Although it is clear that leptin can restore gonadotropic function in hypogonadotropic animals of lean body condition, the means by which this occurs is unknown. The aim of the present studies was to reveal this mechanism, using lean hypogonadotropic sheep. We tested the hypothesis that leptin up regulates POMC gene expression and melanocortin production leading to restoration of reproductive function in lean animals. Our results show leptin can up regulate hypothalamic POMC expression and restore LH pulsatility. Furthermore, MTII given centrally is sufficient to restore LH pulsatility in lean hypogonadotropic ewes. We also present peptide data which shows that acetylated melanocortins in the terminal beds are down-regulated at times of reduced leptin levels. We conclude that central acetylated melanocortin peptides may mediate regulation of reproductive function by leptin in sheep**.**

2.3 Materials and Methods

These experiments were conducted with prior institutional ethical approval under the requirements of the Australian Prevention of Cruelty to Animals Act 1986 and the Code of Practice for the Care and Use of Animals for Scientific Purposes. The animals of this study were inspected by members of the Ethics Committee.

2.3.1 Animals

Adult Corriedale ewes were maintained on pasture or in feedlots, but for intensive experimentation, they were housed individually with natural lighting and temperature and were fed lucerne chaff. Before infusions or sampling, animals were conditioned to pen-housing and handling for 1 week. In experiments where OVX animals were used, ovariectomy was carried out at least one month beforehand, to eliminate cyclic alterations in the secretion of gonadal steroids. Third ventricular (IIIV) and lateral ventricular (LV) cannulations were performed at least two weeks prior to experimentation as previously described (Henry *et al.* 2001a; Henry *et al.* 2008). Animals were made lean by dietary restriction over a period of 6-10 months as described (Henry *et al.* 2000). Briefly, they were fed 500g of pasture hay/day supplemented with straw for bulk. We do not allow our animals to become emaciated, and aimed to achieve body condition scores of 2, indicating a lean condition whereby the ends of the short ribs and backbone can just be felt (Russel 1969). Animals of normal body weight were kept on pasture, with hay supplementation for maintenance. The animals were weighed monthly and adjustments in food intake were made at the discretion of the animal carer, so that target weights of approximately 35kg (lean) and 55kg (normal) were attained. No animal in the lean group was excluded from feeding by dominant flock-mates. When in single pens, lean animals were fed 500g of lucerne chaff/day and normal animals had *ad libitum* access to food. All experiments were carried out in the non-breeding season.

2.3.2 Experiment 1: Effect of leptin on plasma LH levels and expression of NPY, AGRP and POMC in the ARC of lean hypogonadotropic OVX ewes

This experiment was performed to detail the effects of leptin on the expression of key genes in the NPY/AGRP and POMC cells in lean animals, in order to define how leptin may transmit information to the reproductive axis. Lean (33 ± 2.9 kg) OVX ewes (5/group) received IIIV infusions of either 4µg/h human recombinant leptin (Henry *et al.* 2001a) or artificial cerebrospinal fluid (aCSF; 150mM NaCl, 1.2mMCaCl, 1mM MgCl and 2.8 mM KCl) as vehicle at a rate of 55µl/h for 3 days. Blood samples (5 ml) were collected every 10mins for 6h before infusion and for the final 6h of the 72h infusion period. Plasma was harvested and stored at -20°C until assayed for LH. At the end of the infusion period, animals were euthanised by overdose of 20 ml sodium pentobarbital (Lethabarb; Virbac, Peakhurst, NSW, Australia) iv and the heads were removed and perfused through both carotid arteries with 2L normal saline containing heparin (12.5U/ml) followed by 3L of 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4, the final litre containing 20% sucrose. Brains were removed, the hypothalamus dissected and post-fixed at 4°C in fixative containing 30% sucrose for 7 days. Cryostat sections were cut in the coronal plane (20µm), collected into cryoprotectant with 2% paraformaldehyde and stored at -20°C.

In Situ Hybridisation was performed using a ^{35}S dUTP-labelled riboprobe using a described protocol (Simmons DM 1989; Scott *et al.* 2000). The cDNA probes used were (i) a 511 base rat NPY sequence donated by Dr Steven Sabol (National Heart, Blood and Lung Institute, Bethesda, MD, USA) (Estrada *et al.* 2003b) (ii) a 400 base ovine POMC sequence (van de Pavert *et al.* 1997) (iii) a 184 base rat AGRP sequence (Henry *et al.* 2001b). Amplification and linearisation of plasmid DNA was performed using standard technique (Sambrook *et al.* 1989).

For each hybridisation series, 2 sections/ewe were chosen to represent mid and caudal regions of the arcuate nucleus. All cRNA probes were synthesised using a Promega Gemini System II kit (Promega, Annandale, NSW, Australia). Following hybridisation, slides were dipped in Ilford K5 photographic emulsion (Ilford Imaging, Melbourne, VIC, Australia) and kept at 4°C in the dark for either 11 days

(NPY) or 7 days (POMC and AGRP). Image analysis was carried out on autoradiographs using coded slides and the operator was blind to the treatments. As previously described (Henry *et al.* 2001b), the number of silver grains/cell was estimated in 40 cells/section. Cells were counted when silver grain density was >5 times background and when there was a clearly discernible nucleus.

2.3.3 Experiment 2. Effects of MTII and Naloxone on plasma LH levels in normal and lean OVX ewes

The results of experiment 1 showed up regulation of the POMC gene in lean hypogonadotropic ewes, so further evaluation of the melanocortin axis and the opioidergic axis was undertaken. Melanocortins stimulate the reproductive axis (Watanobe *et al.* 1999a), so restoration of POMC expression and production of melanocortins by leptin could be a means by which the reproductive axis is normalised in lean animals. To test this hypothesis we determined whether MTII (MC3-R/MC4-R receptor agonist; Phoenix Pharmaceuticals, CA) would restore pulsatile LH secretion, as a reflection of GnRH secretion. Using a cross-over design, lean (36.5±0.73kg) OVX ewes (n=9) received either MTII (10µg/h) or aCSF (LV) for 3h in alternate weeks. Blood samples (5ml) were collected every 10 min for 3h prior to and 3h during the infusion. Plasma was harvested and stored at -20°C until assayed for LH.

Earlier work (Recabarren *et al.* 1990) suggested that the lean condition may increase negative opioid tone on the reproductive axis. Because the results of experiment 1 led to a focus on the products of the POMC gene, and the transcript of this gene encodes β-END, we investigated this regulatory mechanism. To determine if the reproductive axis was restrained by negative opioid action in the lean condition we administered the non-specific opioid antagonist naloxone. Naloxone was administered first to normal weight animals to determine the efficacy of a dose of 50µg/h using animals of 55.5±0.5kg. Groups received either naloxone (n=5) or aCSF (n=4) for 3 h by LV infusion in a cross-over design. Since the response to naloxone is steroid dependent (Horton *et al.* 1989), the experiment was replicated in OVX ewes with estradiol-17β and progesterone replacement to maximise the chance of an LH response. The former was administered by subcutaneous implantation of 3cm implants inserted into the axillary region for 1

month and intravaginal progesterone delivery devices (CIDR: Riverina; Artificial Breeders Ltd., Albury, Australia) were inserted 2 days prior to the study. Naloxone causes a transient response (Horton *et al.* 1989) which is limited to the first LH pulse following initiation of treatment. This was observed in the steroid-treated animals of normal body weight, so we then repeated the experiment in lean (34.52±0.60kg) animals with and without sex steroid replacement. Since no response was obtained at this naloxone dose, we tested the hypothesis that opioid tone is increased in the lean animals and we infused a 10-fold higher dose (500ug/h) to lean (37.6kg ± 2.1kg) and OVX ewes (n=8) treated with sex steroids, using the same protocol as above.

2.3.4 Experiment 3. Effect of leptin treatment on plasma LH levels and NPY, α-MSH and β-END peptide concentrations in the ARC of OVX lean hypogonadotropic ewes

Leptin treatment of lean hypogonadotropic ewes led to increased POMC gene expression in the arcuate nucleus and restoration of pulsatile LH secretion. In order to determine that the change in gene expression was translated into a change in peptide levels, we undertook an experiment to measure levels of NPY, α-MSH and β-END peptide. Lean (36.6±2.1kg) and normal (63.3±6.1kg) OVX Corriedale ewes were given LV infusions as follows: Lean; aCSF (n=7 for LH analysis, 5 for peptide analysis as a result of damaged tissue), Lean; leptin (n=4), Normal; aCSF (n=4). Leptin (50µg/h) and aCSF was infused at a rate of 55µl/h for 72h with a loading dose of 150µg/165µl. Blood samples (5 ml) were collected every 10mins for 4h prior to the commencement of infusions and 4h afterwards. Further 10 min blood samples were collected between 20-24h and 68-72h of infusion period. Plasma was harvested and stored at -20°C until assayed for LH.

Animals were euthanised at the end of the infusion period and hypothalami dissected and snap frozen on dry ice and stored at -80°C. The blocks were sectioned at 200µm on a cryostat and the ARC dissected with the median eminence removed. Ten sections were collected and placed in 700µl 0.5M acetic acid, boiled for 10min and homogenised. The extracts were centrifuged at 7,500rpm at 4°C for 30min and supernatant collected for HPLC and α-MSH enzyme immunoassay (EIA) analysis.

2.3.5 Experiment 4. Quantification of α-MSH in terminal beds of normal weight and lean ewes

The effect of leptin on the levels of α-MSH in the ARC of lean hypogonadotropic OVX ewes were not as marked as we expected, so further studies were undertaken to measure levels of the peptide in regions of the brain where dense α-MSH-immunoreactive terminal beds are observed. This study was performed on lean and normal weight OVX animals (n=4/group), which represented leptin-deficient and leptin-replete states respectively. The animals were euthanised and the ARC (median eminence removed), hypothalamus (ARC removed) and POA were collected by fresh dissection and snap frozen on dry ice and stored at -80°C. Each tissue region was then extracted *(vide supra)* and quantified for Des-acetylated α-MSH (Des-α-MSH) and acetylated α-MSH (act-α-MSH) using HPLC and α-MSH EIA.

2.3.5.1 NPY Enzyme immunoassay (EIA)

NPY in ARC extracts were assayed using a competitive binding EIA. Plates were first coated with anti-NPY serum 1:25000 (Peninsula Laboratories, San Carlos CA) in EIA assay buffer (1.42g Na_2HPO_4/0.2g KH_2PO_4/5.0gBSA/1ml Tween 20 in water pH7.4) over night at 4°C. The plate was then washed and blocked (8g/l NaCl, 1.42g/ KH_2PO_4, 0.2g/ KCl, 5.0g/l BSA, pH 7.4) for 1h at room temperature. After washing, standards and samples were added at 100µl/well for 1h at room temperature. After removing the supernatant, 100µl of biotinylated NPY 1:500,000 (Bachem, Torrance, CA) was added to all wells except blank and non specific binding wells for 1h. The plate was washed and 100µl of streptavidin-HRP (BD Pharmingen, Franklin Lakes, NJ) was added for 1h. Finally, after 3 washes, 100µl tetramethylbenzidine (TMB) (Pierce, Rockford, IL) was added to each well until a strong blue reaction developed in the total binding wells (10-15 mins). The reaction was terminated by the addition of 100µl 2N H_2SO_4 and the plates were read at 450nm. The sensitivity of this assay was 8pg/100µl.

2.3.5.2 β-END Radioimmunoassay

β-END concentrations in ARC extracts (1:100) were measured in duplicate as previously described (McCarthy *et al.* 1991). β-END (Bachem, Bubendorf, Switzerland) was iodinated using the chloramine T procedure (Greenwood *et al.*

1963) and purified by reverse phase chromatography using a C18-sep-pak column equilibrated with 80%MeOH and 1% FA. This assay had a sensitivity of 10pg/ml.

2.3.5.3 α-MSH Separation and EIA

The α-MSH peptides were separated by HPLC using a Symmetry C18 (5µm) column (4.6 × 150mm) (Waters, Milford, MA). The mobile phase was trifluoroacetic acid (0.1%) acetonitrile:methanol (80:20) gradient, with a flow rate of 1ml/min. The α-MSH peptides eluted between 25-30% acetonitrile:methanol. Fractions (1 ml) were dried and re-hydrated with 50µl of EIA buffer (1.42g/l Na_2HPO_4, 0.2g/l KH_2PO_4, 5g/l BSA, 1ml/l Tween 20, pH 7.4) and assayed to identify the different forms of α-MSH using EIA. The protocol was the same as for the NPY EIA, but 50µl volumes were used. Standards were acetylated α-MSH (Bachem, Torrance, CA), antiserum was Sheep anti-α-MSH (1:25000; Bachem, Bubendorf, Switzerland), and the label was biotinylated α-MSH (1:500,000; Bachem, Bubendorf, Switzerland). Final reaction with TMB was 15-20 min. Assay sensitivity was 4pg/100µl.

2.3.5.4 LH radioimmunoassay

LH in plasma was measured as previously described (Lee *et al.* 1976) using NIH-oLH-S18 as standard and (NIDDK-anti-oLH-I) as antiserum. Iodinated ovine LH (^{125}I-NIDDK-AFD-9598B) was used as tracer. Assay sensitivity was 0.2ng/ml and inter-assay coefficient of variation (CV) was less than 15%.

An LH pulse was defined as having occurred when the assay value of a given sample exceeded the assay value of the previous sample by at least 3 standard deviations, as well as other criteria detailed previously (Clarke and Cummins 1985b) . This method uses error estimates generated by the computer program of Burger *et al.* (Salamonsen *et al.* 1972). LH pulse amplitude was calculated as the difference between the peak pulse and the pre-pulse nadir. LH inter-pulse interval was the average time in minutes, between two successive LH peaks. Mean pre-pulse nadir was calculated as the mean of the lowest hormone value preceding an identified pulse.

Hypogonadotropic condition was defined if one of the following criteria was met: 1. Mean plasma LH level less than 50% of that of the average plasma LH level observed in animals of normal body weight. 2. Plasma LH pulse amplitude less than 50% than the average plasma LH amplitude in animals of normal body weight. 3. Plasma LH pulse frequency greater than 50% of the average plasma LH frequency in animals of normal body weight

2.3.5.5 Leptin radioimmunoassay

Leptin was measured as previously described (Blache *et al.* 2000). The sensitivity of the assay was 0.12ng/ml and the intra-assay CV was less than 10%.

2.3.6 Statistics

Data are presented as means (±SEM). Hormone data were analysed by repeated measures ANOVA, with Least Significant Differences as a *post-hoc* test. Peptide and mRNA levels were analysed by one-way independent measures ANOVA with Tukey's *post-hoc* comparison.

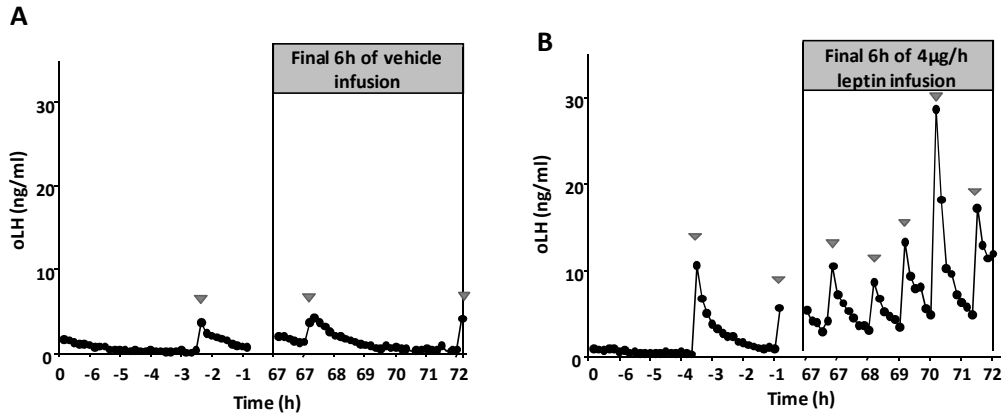
2.4 Results

2.4.1 Leptin levels

Leptin levels were lower ($p<0.05$) in lean animals (0.89±0.1ng/ml) than in animals of normal weight (2.4±0.4ng/ml).

2.4.2 Experiment 1: Effect of IIIv infusion of leptin on plasma LH, and leptin levels and expression of NPY, POMC and AGRP genes.

Our model of long-term reduced body weight produces a range of LH responses as shown previously (Szymanski *et al.* 2007). Infusion of aCSF vehicle had no effect on mean plasma LH levels (Figure 2.1 A,C), but leptin infusion increased mean LH concentrations ($p<0.01$), LH pulse amplitude ($p<0.01$) and LH pre-pulse nadir ($p<0.01$) in lean hypogonadotropic OVX ewes (Figure 2.1 B,C).



C

	LH mean concentration (ng/ml)	LH pulse amplitude (ng/ml)	LH inter-pulse interval (min)	LH pre-pulse nadir (ng/ml)
Lean + aCSF				
Pre-treatment	1.9 ± 1.4	4.7 ± 1.7	150.0 ± 30.0	0.76 ± 0.6
Post-treatment	1.8 ± 0.7	4.8 ± 1.4	120.0 ± 32.1	1.1 ± 0.5
Lean + MTII				
Pre-treatment	2.8 ± 0.6	3.9 ± 1.0	130.0 ± 19.1	2.1 ± 1.3
Post-treatment	9.8 ± 1.2**	10.2 ± 1.7**	69.0 ± 28.3	5.2 ± 0.9**

Figure 2.1: The effect of third ventricular infusion of aCSF **(A)** or leptin (10µg/h; **B**) for 72 h on plasma LH levels in lean, hypogonadotropic OVX ewes. 10min blood samples were collected for 6h prior to the infusion and for the final 6h of the infusion period. **(C)** Analysis of mean LH concentration (ng/ml), LH pulse amplitude (ng/ml), LH inter-pulse interval (min) and LH pre-pulse nadir (ng/ml). Arrow head indicates an LH pulse. **p<0.01 compared to pre-infusion values

Leptin treatment increased expression of POMC (Figure 2.2 A-F) by increasing ($p<0.05$) the number of detectable cells and the level of expression/cell ($p<0.05$). Leptin treatment had no effect on the level of expression of NPY (Figure 2.2 G-L) and AGRP genes (Figure 2.2 M-R).

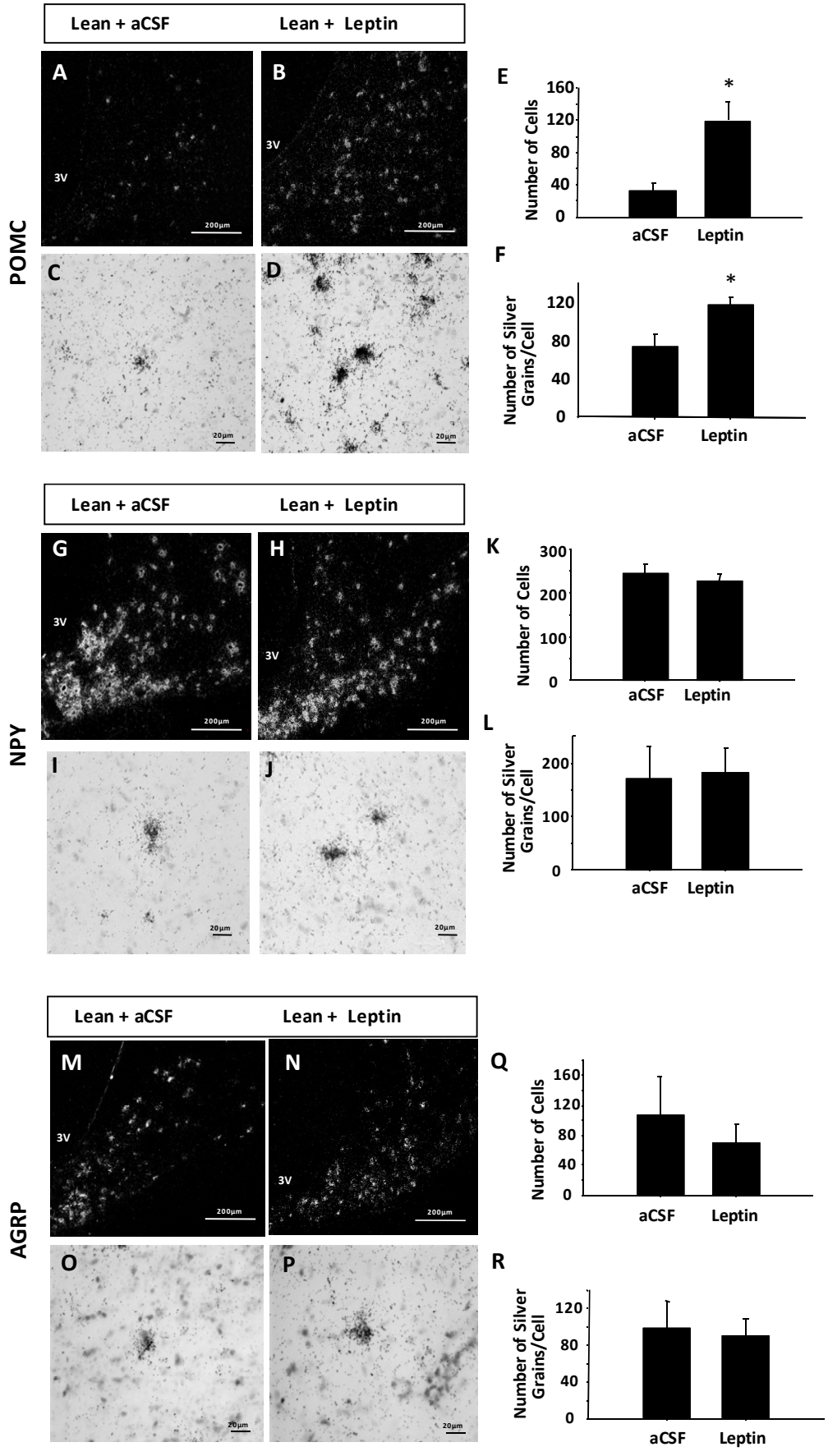
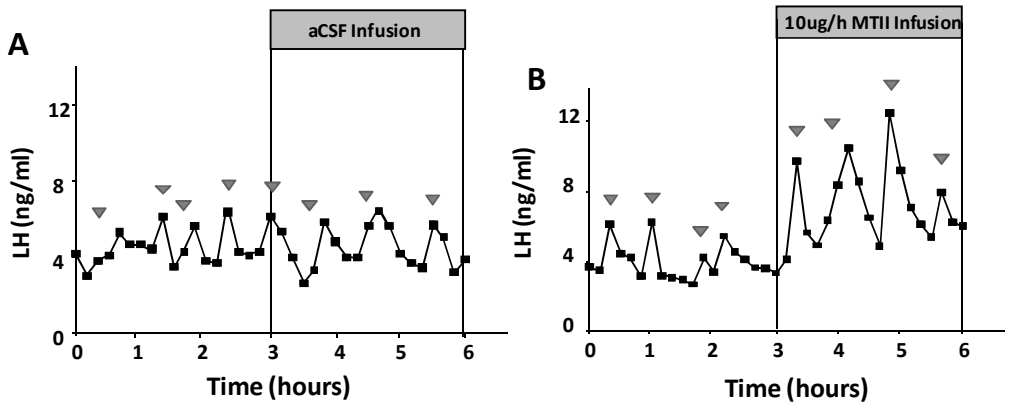

Lean + aCSF
Lean + Leptin
POMC
A
B
C
D
3V
200µm
20µm
E
Number of Cells
160
120
80
40
0
aCSF
Leptin
*
F
Number of Silver Grains/Cell
120
80
40
0
aCSF
Leptin
*
Lean + aCSF
Lean + Leptin
NPY
G
H
I
J
3V
200µm
20µm
K
Number of Cells
300
200
100
0
aCSF
Leptin
L
Number of Silver Grains/Cell
200
100
0
aCSF
Leptin
Lean + aCSF
Lean + Leptin
AGRP
M
N
O
P
3V
200µm
20µm
Q
Number of Cells
160
120
80
40
0
aCSF
Leptin
R
Number of Silver Grains/Cell
120
80
40
0
aCSF
Leptin

Figure 2.2: Effect of third ventricular infusion of aCSF or leptin (50µg/h) for 72 h on NPY, POMC and AGRP gene expression in the arcuate nucleus of the hypothalamus of lean hypogonadotropic OVX ewes, as determined by *in situ* hybridisation. **(A-F)** POMC gene expression in lean control (aCSF) and lean leptin treated hypogonadotropic ewes. **(G-L)** NPY gene expression in lean control (aCSF) and lean leptin treated hypogonadotropic ewes. **(M-R)** AGRP gene expression in lean control (aCSF) and lean leptin treated hypogonadotropic ewes. Darkfield and brightfield photomicrographs are taken at 10X and 40X magnification respectively. Data are means ±SEM. *p<0.05 compared to control.

2.4.3 Experiment 2: Effect of lateral ventricular infusion of MTII and naloxone on plasma LH levels in normal and lean hypogonadotropic ewes

Lateral ventricular infusion of MTII increased mean LH concentration (p<0.01), LH pulse amplitude (p<0.05), and LH pre-pulse nadir (p<0.01), compared to pre-treatment LH levels in lean, hypogonadotropic OVX ewes. Vehicle treatment had no effect. In contrast to the response to leptin, the response to MTII occurred immediately upon the commencement of infusion (Figure 2.3).

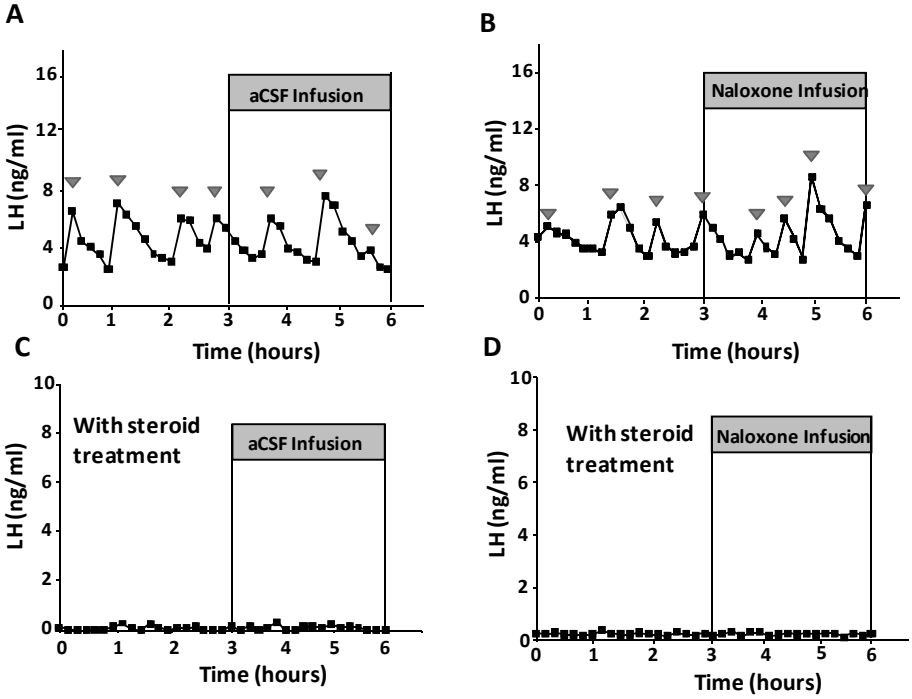


C

	LH mean concentration (ng/ml)	LH pulse amplitude (ng/ml)	LH inter-pulse interval (min)	LH pre-pulse nadir (ng/ml)
Lean + aCSF				
Pre-treatment	5.7 ± 1.5	3.5± 0.7	33.0± 3.4	4.5± 1.3
Post-treatment	5.8 ± 1.5	3.9± 0.9	29.1± 3.5	3.9± 0.7
Lean + MTII				
Pre-treatment	5.5± 1.6	3.5± 0.9	35.14± 3.3	4.1± 1.3
Post-treatment	9.1± 2.3**	9.4± 1.7*	32.8± 2.3	9.7± 1.6**

Figure 2.3: The effect of lateral ventricular infusion of aCSF **(A)** or melanocortin agonist MTII (10µg/h; **B**) for 3 h on plasma LH levels in lean hypogonadotropic OVX ewes. **(C)** Analysis of mean LH concentration, LH pulse amplitude, LH inter-pulse interval (min), and LH pre-pulse nadir. Arrow head indicates an LH pulse. *P<0.05 and **p<0.01 compared to pre-treatment values.

Naloxone (50µg/h) increased the amplitude of the first LH pulse following the onset of infusion ($p<0.01$) in steroid-treated controls (data not shown). The same treatment had no effect on plasma LH levels in lean, hypogonadotropic OVX ewes treated with or without gonadal steroids (Figure 2.4). The higher dose of 500µg/h naloxone did not increase plasma LH levels in lean animals (data not shown).



E

	LH mean concentration (ng/ml)	LH pulse amplitude (ng/ml)	LH inter-pulse interval (min)	LH pre-pulse nadir (ng/ml)
Lean + aCSF				
Pre-treatment	5.9 ± 1.6	4.4 ± 0.7	37.5 ± 3.1	2.9 ± 1.2
Post-treatment	5.6 ± 1.9	3.7 ± 1.3	33.8 ± 5.6	4.2 ± 1.7
Lean + Naloxone				
Pre-treatment	4.1 ± 1.7	2.8 ± 0.5	32.5 ± 4.3	1.6 ± 0.6
Post-treatment	4.2 ± 2.1	2.8 ± 0.5	34.6 ± 7.2	2.9 ± 0.7
Lean + P + E + aCSF				
Pre-treatment	0.2 ± 0.01	0.1 ± 0.0	180 ± 0.0	0.1 ± 0.0
Post-treatment	0.2 ± 0.03	0.1 ± 0.0	180 ± 0.0	0.1 ± 0.0
Lean + P + E + Naloxone				
Pre-treatment	0.08 ± 0.01	0.1 ± 0.0	180 ± 0.0	0.1 ± 0.0
Post-treatment	0.45 ± 0.35	0.07 ± 0.02	180 ± 0.0	0.07 ± 0.02

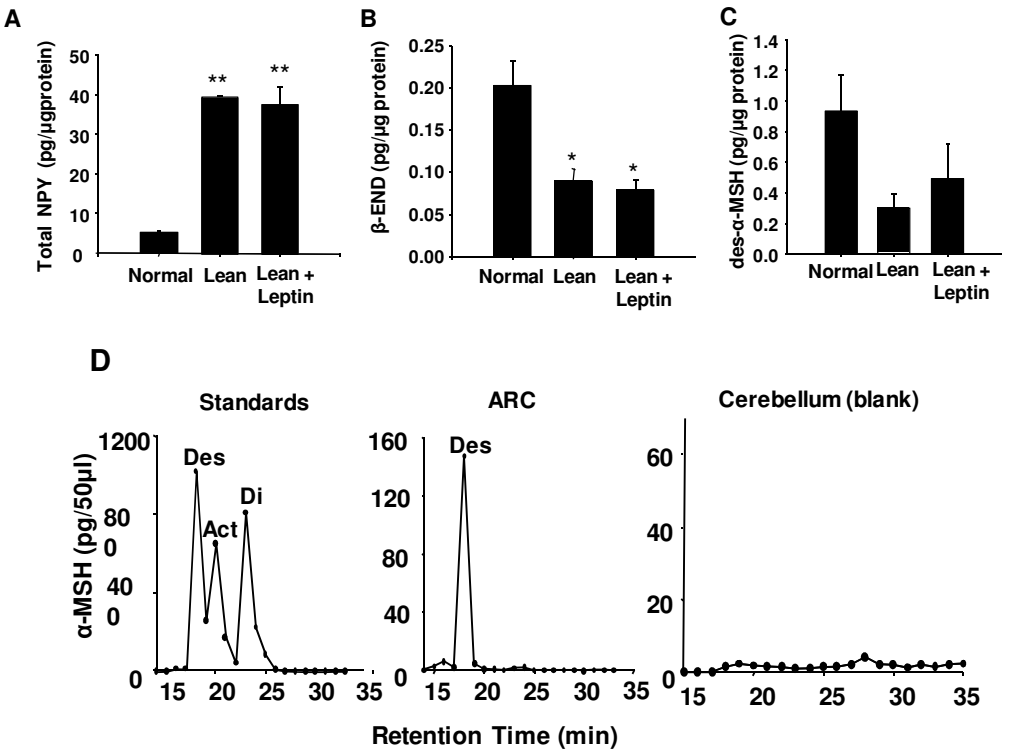
Figure 2.4 The effect of lateral ventricular infusion of aCSF or naloxone for 3 h with **(A,B)** or without **(C,D)** sex steroids, estrogen (E) and progesterone (P), on plasma LH levels in lean hypogonadotropic OVX ewes. **(E)** Analysis of mean LH concentration, LH pulse amplitude, LH inter-pulse interval (min), and LH pre-pulse nadir. Arrow head indicates an LH pulse.

2.4.4 Experiment 3: Effect of ICV administration of leptin on NPY, β-END and post-translationally modified forms of α-MSH in the ARC of lean, hypogonadotropic OVX ewes.

Plasma levels of LH were lower in the lean animals than in the normal animals (5.6±0.36ng/ml) prior to treatment. Although the mean plasma LH level and the plasma LH pulse amplitude were the same before treatment in the lean vehicle-treated and leptin-treated animals, the inter-pulse interval was significantly higher ($P<0.05$) in the latter group. This was because 3 of the animals in the leptin-treated group had no discernable LH pulses in the pre-treatment period.

NPY peptide levels were significantly ($p<0.01$) higher in lean animals than in normal animals (Figure 2.5 A). Levels of β-END were significantly ($p<0.05$) lower in lean ewes (Figure 2.5 B) and des-α-MSH tended ($p<0.06$) to be lower (Figure 2.5 C). Leptin treatment did not significantly alter the levels of NPY, β-END or des-α-MSH.

Des-α-MSH was the only form of α-MSH detected in the ARC and no α-MSH of any form was detected in the blank tissue (cerebellum; Figure 2.5 D). Leptin treatment to these animals significantly increased mean LH concentration for the final 4h of infusion ($p<0.05$), and increased LH pulse amplitude in between 20-24h of infusion ($p<0.05$) and between 68-72h of infusion ($p<0.01$). LH inter-pulse interval was decreased following leptin treatment between 20-24h and 68-72h of infusion ($p<0.01$; Figure 2.5 E).



E

	LH mean concentration (ng/ml)	LH pulse amplitude (ng/ml)	LH inter-pulse interval (min)	LH pre-pulse nadir (ng/ml)
Lean + aCSF				
-4 - 0h aCSF	3.33 ± 0.72	2.13 ± 0.48	91.05 ± 21.53	2.57 ± 0.66
0-4h aCSF	3.29 ± 0.88	1.73 ± 0.41	84.17 ± 27.84	2.33 ± 0.79
20-24h aCSF	3.62 ± 0.92	2.86 ± 0.63	100.0 ± 14.29	2.51 ± 0.74
68-72h aCSF	3.45 ± 0.89	2.64 ± 0.74	93.57 ± 29.25	2.50 ± 0.07
Lean + Leptin				
-4 - 0h aCSF	1.65 ± 0.82	1.28 ± 1.1	200.0 ± 40.0†	1.38 ± 0.63
0-4h aCSF	1.63 ± 0.99	1.09 ± 1.0	195.8 ± 44.2	1.30 ± 0.67
20-24h aCSF	3.38 ± 1.10	6.35 ± 3.1^	136.2 ± 42.2^^	1.88 ± 0.77
68-72h aCSF	4.94 ± 1.13^	9.03 ± 2.13^^	121.6 ± 30.6^^	2.87 ± 0.96
Normal + aCSF				
-4 - 0h aCSF	8.45 ± 2.46	4.85 ± 1.37	47.62 ± 3.03	6.54 ± 2.01
0-4h aCSF	7.57 ± 2.04	4.06 ± 0.86	49.31 ± 5.91	5.98 ± 1.80
20-24h aCSF	8.18 ± 2.84	4.75 ± 1.77	43.56 ± 2.87	6.11 ± 2.04
68-72h aCSF	7.51 ± 2.51	5.25 ± 2.24	48.66 ± 2.86	5.52 ± 1.89

Figure 2.5: Mean peptide levels of NPY **(A)**, des-α-MSH **(B)**, and β-END **(C)**, in the ARC of normal, lean, and lean leptin-treated (50µg/h) OVX ewes. All data are presented as mean ±SEM. **(D)** HPLC/EIA profile of MSH standards, des-α-MSH, act-α-MSH and Di-act-α-MSH and representative HPLC/EIA profiles in the ARC and Cerebellum. **(E)** Analysis of mean LH concentration (ng/ml), LH pulse amplitude (ng/ml), LH inter-pulse interval (min) and LH pre-pulse nadir (ng/ml) presented as mean ±SEM. *p<0.05 and **p<0.01 compared to ewes of normal body weight. ^p<0.05 and ^^p<0.01 compared to pre-treatment -4-0h LH values. †p<0.05 compared to lean -4-0h LH inter-pulse interval

2.4.5 Experiment 4: Quantification of des-α-MSH and act-α-MSH in the terminal beds of normal and lean ewes

Des-α-MSH tended ($p<0.059$) to be lower in the ARC of lean ewes. Act-α-MSH was detected in the ARC of 2/4 normal animals, leading to a large standard error for measures in this nucleus (Figure 2.6 A). Act-α-MSH levels were lower in the terminal beds of the hypothalamus (ARC removed; $p<0.05$; Figure 2.6 B) and the POA ($p<0.01$; Figure 2.6 C) of lean hypogonadotropic ewes.

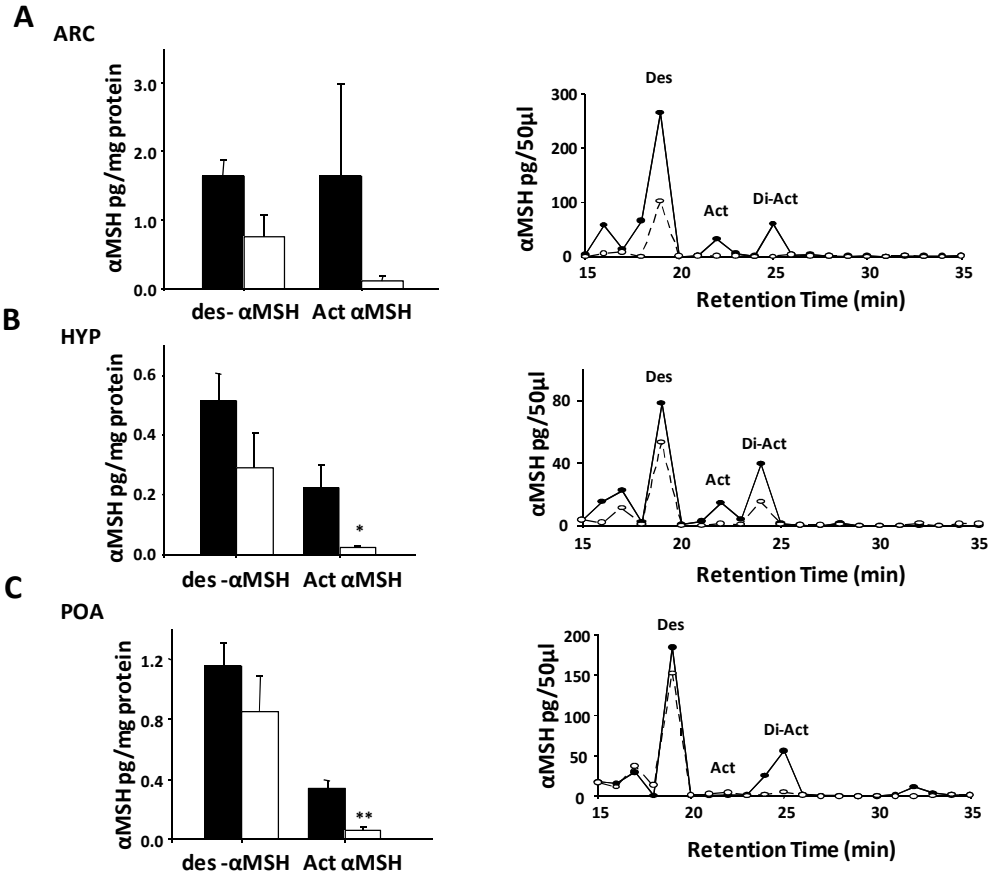


Figure 2.6: Mean (±SEM) peptide levels of des-αMSH and act-αMSH in the ARC **(A)**, HYP (ARC removed) **(B)**, and POA **(C)** of normal weight (solid bar) and lean hypogonadotropic (open bar) ewes and HPLC/EIA representative profiles for each nuclei (*Continuous* line represents normal weight ewes and *dotted* line represents lean hypogonadotropic ewes). *p<0.05 and **p<0.01compared to normal weight controls.

2.5 Discussion

POMC gene expression is reduced and NPY gene expression is increased with reduction in body weight, in association with a hypogonadotropic state (McShane *et al.* 1993). Central leptin infusion to the brain restores pulsatile LH secretion and this is associated with an up regulation of POMC expression, but no change in NPY expression. Because pulsatile LH secretion reflects GnRH secretion (Clarke and Cummins 1982), we can confidently use the former as an index of the latter. In addition to the response of the POMC gene to leptin treatment of lean, hypogonadotropic OVX ewes, we show that treatment of lean animals with a melanocortin agonist, MTII, can restore pulsatile LH secretion. This lends support to the notion that the means by which leptin stimulates GnRH/LH secretion may be via the POMC cells and the production of melanocortins. Act-α-MSH levels in regions of the brain where melanocortin terminal beds are found, viz 'non-arcuate' hypothalamus and POA, were lower in lean hypogonadotropic ewes. This strongly suggests that MTII mimics the effects of act-α-MSH and activates neurons outside of the ARC to stimulate the reproductive axis.

Leptin treatment is able to restore GnRH and gonadotropin levels in rats (Watanobe 2002) and monkeys (Finn *et al.* 1998) following 2-3 days starvation, but our model is one in which a hypogonadotropic state occurs due to a reduction in body reserves over a long period of time. This is important because unlike the acute challenge presented to starved laboratory rats, our animals establish an altered metabolic set point to normal animals and are not compromised by health. In the long term food restricted sheep, leptin treatment restores gonadotropin secretion (Henry *et al.* 2001a), but re-feeding restores LH secretion without a change in leptin levels (Szymanski *et al.* 2007), which may be due to altered leptin transport upon re-feeding. Alternatively, the relevant cells in the brain may become more responsive to leptin with re-feeding, but this remains to be determined. Certainly, leptin transport in sheep can change markedly with season (Adam *et al.* 2006), so it is possible that it also changes with body weight in sheep. ARC Ob-Rb mRNA is up regulated in lean sheep but its expression within POMC cells is unchanged with reduced body weight (Kurose *et al.* 2005). Notwithstanding these issues, it is clear from earlier studies and the present work that central leptin

infusion restores pulsatile LH secretion in our lean, hypogonadotropic model, suggesting that the subcellular signalling machinery for leptin action is operative.

POMC mRNA levels are reduced and NPY and AGRP mRNA levels are increased in lean fasted rats (Korner *et al.* 2001) and in sheep subjected to chronic food restriction (McShane *et al.* 1993; Henry *et al.* 2001b). In some studies, however, this effect of body weight on POMC expression has not been observed, perhaps due to variation between animals (Henry *et al.* 2000). In laboratory rats, leptin treatment is able to reverse the fasting induced changes in NPY, AGRP and POMC gene expression (Korner *et al.* 2001). The results of the present study yielded a different result, since leptin treatment increased POMC expression without altering either NPY or AGRP expression. For this reason, we focussed further on the POMC system.

It seems likely that NPY is elevated in lean ewes as a compensatory mechanism to increase appetite drive. It is possible that leptin cannot restore levels to normal without an increase in body weight because other metabolic factors signal to the NPY cells. The present result concurs with our earlier studies (Henry *et al.* 2001a) showing that central leptin treatment restores LH levels in lean OVX ewes without reducing food intake. This substantiates the notion that NPY expression remains high in these animals to maintain appetite drive until body weight is corrected to normal. The earlier study also showed that treating OVX ewes of normal weight with leptin had the opposite effect, having no effect on plasma LH levels, but reducing food intake (Henry *et al.* 2001a). Collectively, these point towards a dissociation between the means by which leptin affects food intake and reproductive function respectively.

There is evidence to suggest that an increase in endogenous opioid tone, created by endorphins, enkephalins, dynorphins and endomorphins, is responsible for the suppression of pulsatile LH secretion at times of reproductive quiescence (Leadem and Kalra 1985). Consistent with our investigation of the role of the POMC system in the control of reproductive function, especially in the lean condition, we considered it important to entertain the notion that opioids (specifically β-END) might be involved. This could occur by preferential increase in β-END production

(through differential post-translational processing in favour of β-END) and/or increased opioid receptor expression. We present evidence that there is no selective increase in the production of β-END peptide, and that enhanced opioid tone is unlikely since a dose of the non-specific opioid antagonist, naloxone, or 10 times this dose, did not increase LH secretion in the hypogonadotropic, lean animals. This agrees with, and extends the results of others (Recabarren *et al.* 1990) who studied food-restricted ewe lambs. There are two possible interpretations of these data. One is that the opioid system has no role to play in the reduction of GnRH/LH secretion in the hypogonadotropic, lean condition. The other is that there is profoundly enhanced opioid tone. To fully interrogate this aspect of the lean condition, it seems most appropriate to examine the role of the dynorphin and enkephalin systems, both of which appear to play an important role in the control of GnRH neurons (Walsh and Clarke 1996; Goodman *et al.* 2004), but this was beyond the scope of the present study, which focussed on the POMC neurons. An indication that altered production of β-END is not the means by which leptin transmits information to the GnRH cells is that leptin treatment did not affect the production of β-END peptide, even though the same treatment increased POMC expression and pulsatile LH secretion.

On the other hand, MTII restored LH secretion, strongly indicating a role of the melanocortins in the control of the reproductive system at times of compromised energy availability. This notion is supported by rat (Watanobe *et al.* 1999a) and human (Limone *et al.* 1997; Stanley *et al.* 2003) studies showing inhibition or stimulation of LH pulsatile secretion following administration of a melanocortin antagonist or agonist respectively. We found that MTII acted much faster than leptin in restoring pulsatile LH secretion. Thus, we speculate that the melanocortins act downstream of leptin, and this slower response with leptin indicates slower transcriptional or post-translational events required for leptin to act. Our results are consistent with MTII acting on downstream effector cells, bypassing leptin-mediated effects on POMC cells. In support of this notion are the discoveries that almost all POMC cells in the ovine ARC express the leptin receptor Ob-Rb (Iqbal *et al.* 2001b), and ovine POMC cells are activated following intravenous leptin injection (Clarke, unpublished data). Additionally, α-MSH

(Limone *et al.* 1997) and γ-MSH (Stanley *et al.* 2003) are capable of stimulating LH secretion in humans, and central melanocortin receptor antagonist can reduce the effectiveness of leptin to restore gonadotropin secretion in starved rats (1999a). It does however remain plausible that MTII elicits its effect independent of leptin signalling to rescue GnRH/LH secretion. Many neuronal systems have been implicated in the regulation of GnRH secretion (Clarke and Pompolo 2005), and the possible involvement of melanocortins does not preclude effects of other systems. It is probable that the melanocortins communicate with numerous different cells types to stimulate the reproductive axis. One possibility is the recently discovered peptide kisspeptin, since Kiss1 mRNA is down-regulated at times of reduced energy stores, and treatment with kisspeptin is able to restore vaginal opening and elicit gonadotropin and estrogen responses (Castellano *et al.* 2005). Melanocortins may interact with kisspeptin in lean hypogonadotropic states to stimulate the reproductive axis downstream to leptin or independently.

In agreement with our observation that NPY gene expression is strongly up regulated in the lean condition, NPY peptide levels were also increased and leptin treatment was unable correct this towards normal levels. Although NPY is a negative regulator of the reproductive axis in the ewe (Clarke *et al.* 2005), the gene expression data and the peptide data presented herein suggest that the GnRH/LH response to leptin treatment in lean hypogonadotropic animals is not explained by a reduction in the level of this peptide.

Many studies have focused on POMC gene transcription and how this may or may not change with body weight (McShane *et al.* 1993; Mizuno *et al.* 1998; Henry *et al.* 2001b), but little is known of the peptides produced by post-translational processing of the precursor. Thus, it was considered important to ascertain whether there was a differential shift in the processing of the precursor (β-END vs melanocortins). In agreement with data from the rat (Guo *et al.* 2004), we show that des-α-MSH is the predominant form of α-MSH detected in the ARC, but acetylated forms were found in two of the normal weight animals from experiment 4. This is likely due to the difference in the dissection methods used in experiments 3 and 4. In experiment 3, the arcuate nucleus was removed by microdissection of sectioned tissue, but in experiment 4 we wished to examine

tissues in which melanocortin terminal beds are found. Rather than micro-dissecting very specific regions, we undertook to determine whether there was a generalised difference in the type of melanocortin found in the region of the perikarya of POMC cells (ARC) and areas to which these cells project. In experiment 3, using the more precise dissection method, we showed that ARC samples from lean animals contained des-α-MSH only. The presence of acetylated forms of the peptide in the ARC samples of experiment 4 was most likely due to the inclusion of some non-ARC tissue in these samples. We conclude that des-α-MSH is the major form of α-MSH in the ARC and that the peptide is acetylated shortly before export from the cell body or in the axons or terminals of the neurons. There was a trend (P=0.06) towards reduced des-α-MSH peptide levels in lean hypogonadotropic conditions in the ARC, and levels in leptin-treated animals were midway between those in normal animals and those in lean animals (not statistically significant). Further work on the acetylation of this and other peptides in the brain is in progress.

Projections of melanocortin-producing neurons, terminal beds (Jegou *et al.* 2003) and receptors (Mountjoy *et al.* 1994) are found throughout the brain. Measurement of the level of function of these neurons will require quantification of the level of peptide that is produced and exported to the neuronal targets. In particular, measuring POMC gene expression and/or des-α-MSH in the ARC may not indicate the full profile of the melanocortin status of the brain. It has been suggested that leptin acts to increase N-acetyltransferase activity, increasing the amount of the more potent act-α-MSH (Tsujii and Bray 1989; Guo *et al.* 2004), and that α-MSH may be acetylated *en passage* to terminal beds (Bunel *et al.* 1990). This is substantiated in the current study, but it would be instructive to identify the enzyme and determine what regulates its activity. Although des-α-MSH is the predominant form in all nuclei analysed, we believe that the salient changes are seen in the acetylated forms. Another consideration is that change in function of POMC-derived melanocortins is brought about by a change in MC3 and MC4R expression in the brain, but our earlier studies suggest that expression of these receptors is unchanged by alteration of bodyweight in sheep (Iqbal *et al.* 2001a).

In summary, this study suggests that a link exists between energy stores, the melanocortin system and the reproductive axis. We show that leptin can act to increase POMC gene expression in lean hypogonadotropic animals, suggesting that this leads to increased melanocortin production and function. This correlates to increased secretion of LH in lean animals. Activation of the melanocortin receptor with MTII immediately restores LH levels in lean animals, consistent with the hypothesis that the down-regulation of the POMC system is the cause of the hypogonadotropic condition in these animals. Act-α-MSH levels are lower in the POA and in areas of the hypothalamus outside of the ARC in lean ewes with low leptin levels. We speculate that leptin may increase the acetylation of α-MSH during neuronal transport, and this acetylated form may act on target cells in the POA and in areas of the hypothalamus outside of the ARC, leading to an increase in pulsatile LH secretion. Melanocortins may have specific utility in the restoration of reproduction in situations of low body weight, caused by excessive exercise or other conditions.

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Chapter 3

Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe

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Declaration for Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiment management/conduct, laboratory analysis, data collection, statistical analysis, preparation of manuscript	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Iain J Clarke[1]	Provided intellectual input and editing of manuscript	N/A
Jeremy T Smith[1]	Provided intellectual input and editing of manuscript	N/A

Candidate's Signature		Date

Declaration by co-authors

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	[1]Monash University, Department of Physiology, Clayton Campus

Signature 1		
Signature 2		

Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe

3.1 Abstract

To further test the hypothesis that melanocortins stimulate the reproductive axis we treated ewes with a melanocortin agonist (MTII) in the luteal phase of the estrous cycle and during seasonal anestrus. Lateral ventricular (LV) infusion of MTII (10μg/h) during the luteal phase of the cycle increased LH secretion. Retrograde neuronal tracing in the brain showed that few POMC or kisspeptin cells in the arcuate nucleus (ARC) project to the preoptic area (POA) where GnRH cells are located. MTII infusion (20h) was repeated in luteal phase ewes and brains were harvested to measure gene expression of pre-pro-orexin (ORX) and kisspeptin (Kiss1). Expression of ORX in the dorsomedial hypothalamus (DMH) and Kiss1 in the POA was up regulated by MTII treatment and Kiss1 in the ARC was down regulated. Seasonally anestrous ewes were progesterone-primed and then treated (LV) with MTII (10 μg/h) or vehicle for 30h and blood samples were collected every 2h from 4h before infusion until 6h afterwards to monitor acute response in terms of LH levels. A rise in basal LH levels was seen but samples collected around the time of the predicted LH surge did not indicate that an ovulatory event occurred. We conclude that melanocortins are positive regulators of the reproductive neuroendocrine system, but treatment with melanocortins does not fully overcome seasonal acyclicity. The stimulatory effect of melanocortin in the luteal phase of the estrous cycle may be via the activation of kisspeptin cells in the POA and/or orexin cells in the DMH.

3.2 Introduction

Post-translational processing of the proopiomelanocortin (POMC) gene results in the formation of endorphin and melanocortin products in the hypothalamus (Pritchard *et al.* 2002). Whereas POMC mRNA expression in the arcuate nucleus (ARC) appears to be unchanged across the estrous cycle in the ewe (Walsh *et al.* 1998), sex steroid effects on post-translational processing of POMC remains to be determined and differential processing of the POMC precursor peptide could occur. In the rat, POMC mRNA expression is reduced at the time of the pre-ovulatory LH surge (Wise *et al.* 1990; Bohler *et al.* 1991), and treatment of ovariectomised (OVX) rats with estrogen reduces POMC mRNA (Wilcox and Roberts 1985; Tong *et al.* 1990; Petersen *et al.* 1993); progesterone treatment is able to attenuate or reverse this effect (Wilcox and Roberts 1985; Wise *et al.* 1990). On the other hand, others have shown that a reduction in POMC mRNA does not occur in OVX rats when an LH surge is activated by estrogen/progesterone treatment (Petersen *et al.* 1993). These studies were predicated on the notion that ß-endorphin derived from the POMC precursor negatively regulates GnRH cells and that reduction in expression of the precursor gene would reduce opioid tone at the time of the preovulatory GnRH/LH surge.

Melanocortins stimulate the reproductive axis in various species including rats, sheep, and humans (Schioth and Watanobe 2002). For example, α melanocyte stimulating hormone (α-MSH) induced ovulation in female rats pre-treated with progesterone (Alde and Celis 1980). Administration of a melanocortin antagonist reduced the magnitude of the LH surge in OVX steroid primed rats (Watanobe *et al.* 1999a) and treatment of human males with α-MSH increased plasma LH levels (Limone *et al.* 1997). Moreover, gamma-MSH stimulates GnRH release in immortalised GnRH cell lines and hypothalamic explants *in vitro* and plasma LH levels *in vivo* (Stanley *et al.* 2003). Recent studies of lean OVX ewes show that a melanocortin agonist can overcome the hypogonadotropic state of these animals, stimulating pulsatile LH secretion (Backholer *et al.* In Press-a). Because the POMC cells are important in the regulation of energy balance (McShane *et al.* 1993; Mizuno *et al.* 1998), these cells (and the melanocortins) could provide a

mechanism by which energy status and reproduction are linked. Whether these POMC cells synapse directly with GnRH neurons is unknown, however we have shown previously that very few cells from the ARC actually synapse with GnRH neurons in the ventral POA (Pompolo *et al.* 2001). Conversely, others, using retrograde (Goubillon *et al.* 1999) and anterograde (Goubillon *et al.* 2002) tracers, have detected input from the ARC to GnRH cells. These latter studies however report large injection volumes and do not limit the injection sites to the region of interest where GnRH or ARC cells are located, and we believe an inter-neuronal pathway between the ARC cells and GnRH neurons is likely. Orexin (ORX) (Pu *et al.* 1998) and Kisspeptin (Irwig *et al.* 2004; Dhillo *et al.* 2005; Shahab *et al.* 2005; Smith *et al.* 2006b) have both been reported as positive regulators of the neuroendocrine reproductive axis, and both these cell types have demonstrated direct input to GnRH cells in the sheep (Iqbal *et al.* 2001c; Smith *et al.* 2008a) and rodent (Campbell *et al.* 2003). It is therefore possible that these cells act as a conduit for melanocortin feedback, however this is yet to be elucidated.

In order to further investigate the role of melanocortins in the regulation of reproduction, we have carried out studies in the ewe, using a melanocortin agonist Melanotan II (MTII). Firstly, we performed lateral ventricular (LV) infusions of the agonist in ewes during the luteal phase of the estrous cycle, which is a period when gonadotropin secretion is held under negative feedback by the combined effects of estrogen and progesterone (Karsch *et al.* 1987). Since we observed a stimulatory effect of the agonist on plasma LH levels, we then treated animals in the luteal phase of the cycle with the agonist and examined gene expression for pre-pro orexin (ORX) and kisspeptin (Kiss1), because these may act as neurotransmitter intermediaries to regulate the gonadotropin releasing hormone cells (Iqbal *et al.* 2001c; Smith *et al.* 2008a). In order to determine whether melanocortin action can overcome photoperiodic suppression of the reproductive axis, we infused MTII (LV) into seasonally anestrous ewes to examine acute response in terms of LH secretion and possible stimulation of ovulation.

3.3 Materials and Methods

3.3.1 Animals

The experiments used adult Corriedale ewes which were maintained on pasture or in feedlots under natural conditions. For intensive experimentation, the animals were housed in individual pens with natural lighting and had access to lucerne chaff and water *ad libitum.* The animals were conditioned to pen-housing and handling for 1 week prior to experimentation and LV cannulation was carried out at least 2 weeks before experimentation as previously described (Henry *et al.* 2008). All animal procedures were conducted with prior institutional ethical approval of the Animal Experimentation Ethics Committee of Monash University fulfilling the requirements of the Australian Prevention of Cruelty to Animals Act 1986 and the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Animal Commission *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

3.3.2 Experiment 1 – Effect of MTII treatment during the luteal phase of the estrous cycle on LH secretion

Eight adult Corriedale ewes received lateral ventricular (LV) cannulae, as previously described (Henry *et al.* 2008) at least 1 month prior to use. Their estrous cycles were synchronised by 2 sequential intra-muscular injections of 125µg of synthetic luteolysin Cloprostenol (Estrumate; Pitman-Moore, Sydney, Australia) 10 days apart and the experiment was performed 10 days after the second injection (late luteal phase of the estrous cycle). On the day prior to experimentation, the animals received an external jugular venous cannula (Tuta Healthcare, Sydney, New South Wales, Australia) which was kept patent with heparinised (50U/ml) saline. The cannula was extended to the side of the animal pens with polyethylene tubing and closed with a three-way stopcock. Verification of the stage of cycle was by measurement of plasma progesterone levels (Scott *et al.* 2001), which were 4.1 ± 0.7ng/ml. Serial blood sampling commenced at 0900h and samples (5 ml) were taken every 10 min for 3h prior to treatment and for 3h during infusion. MTII (10µg/h; n=4) or artificial cerebrospinal fluid (aCSF) as vehicle (n=4) was administered by LV infusion (55µl/h) using MS16A Grasby mini-pumps (Graseby

Medical Ltd., Gold Coast, Australia). Plasma was harvested and stored at -20°C until assayed for LH.

3.3.3 Experiment 2 – Retrograde tracing from the preoptic area (POA) to the POMC and Kisspeptin cells in the ARC

Retrograde labelling from the POA was performed to determine whether ARC POMC and/or kisspeptin cells project to the preoptic area, where the majority of GnRH cells are found (Lehman *et al.* 1986). Analysis of kisspeptin cells was included because of the possibility that MTII acts via these cells to affect GnRH secretion (*vide infra*). Adult Corriedale ewes (n=4) received 75nl injections of 4% FG (Fluorochrome Inc., Englewood, Colo., USA) into the ventromedial POA as previously described (Iqbal *et al.* 2001c). Animals were then returned to pasture for 3 weeks to allow optimum retrograde transport of FG to the ARC in the brain. After this time, the animals were euthanised by intravenous (i.v) injection of 20ml sodium pentobarbital (Lethabarb; May and Baker Pty. Ltd., Melbourne, Vic., Australia) and the brains were perfused and processed for immunohistochemistry as described previously (Qi *et al.* 2008). Frozen sections were cut in the coronal plane (40μm), collected into cryoprotectant at -20°C until processed. For each animal, 3 sections representing rostral, middle and caudal ARC were processed for fluorescent immunohistochemistry (*vide infra*).

3.3.4 Experiment 3 – ORX and Kiss1 gene expression following MTII treatment

MTII stimulated LH secretion in Experiment 1, but the results of experiment 2 showed that very few POMC or kisspeptin cells projected to the ventral POA. We repeated the treatments of experiment 1 in the same animals to ascertain whether MTII affected expression of genes for neuropeptides that may act as interneurons between POMC cells and GnRH cells, namely kisspeptin cells and ORX cells. Following Experiment 1, the ewes were allowed to progress through another estrous cycle and were in the luteal phase of the estrous cycle when they were randomly assigned to receive LV infusions of either MTII (10μg/h) or vehicle (aCSF) for 20h. This time-frame was chosen to allow adequate time for possible changes in gene expression to occur. The brains of the animals were perfused with

paraformaldehyde as previously described (Henry *et al.* 2001b). The hypothalamus was dissected as a block and post-fixed at 4°C in fixative containing 30% sucrose for 7 days. Frozen sections were cut in the coronal plane (20µm), collected into cryoprotectant with 2% paraformaldehyde and stored at -20°C until processed for *in situ* hybridisation analysis of ORX and Kiss1 mRNA.

3.3.5 Experiment 4 – MTII treatment to acyclic anestrous ewe

MTII treatment stimulated LH secretion in the breeding season so we determined whether the melanocortin agonist could stimulate gonadotropin secretion and induce ovulation in seasonally acyclic anestrous ewes. Adult Corriedale ewes with LV cannulae were treated during November (anestrous season for this breed) and received LV infusions of either MTII (10µg/h; n=6) or vehicle (aCSF; n=6). Prior to the experiment all ewes were treated with progesterone to prime the neuroendocrine system to effects of estrogen (Caraty and Skinner 1999). This was carried out by the insertion of an intravaginal progesterone controlled delivery device (CIDR) (InterAg, Hamilton, New Zealand). The CIDRs were removed and 24h later the animals received either MTII or vehicle (aCSF) for 30h (55µl/h). Jugular blood samples were collected every 2h from 4h before infusion until 6h afterwards to monitor acute response in terms of LH levels. Then, from 28h-35h after the commencement of MTII infusion, additional blood samples were collected hourly, to detect the occurrence of an LH surge. Finally, blood samples were taken every second day for 14 days for assay of plasma progesterone, to determine if ovulation had occurred with an ensuing corpus luteum.

3.3.6 Immunohistochemistry and FG mapping

The FG injection sites were localised using fluorescence microscopy (for FG) on sections counterstained with cresyl fast violet as previously described (Qi *et al.* 2008). Animals with correct placement of FG injections within the POA were selected for further study. Anatomically matching sections representing rostral, middle and caudal ARC from each animal were mounted onto superfrost slides and dried overnight. POMC containing cells were identified using a γ-MSH primary antibody (Antibodies Australia, Melbourne) with specificity as previously described (Goodman *et al.* 2007). Pre-absorption with 0.5mg/ml of the original peptide

abolished all staining in the ovine ARC (data not shown). To detect kisspeptin, we used a polyclonal rabbit antibody against mouse kisspeptin-10 (dilution1:100,000 ; gift from A. Caraty, INRA, Nouzilly, France) (Franceschini *et al.* 2006) previously validated for use in sheep tissues (Goodman *et al.* 2007). Secondary antibodies for γ-MSH and kisspeptin staining were anti-guinea pig and anti-rabbit respectively, conjugated to Alexa 546 (1:500, Molecular Probes, Inc, Eugene, OR). Following staining for γ-MSH or kisspeptin, the sections were then labelled for FG (rabbit polyclonal antibody ; dilution 1:2000; Chemicon International, Sydney, Australia) using a goat anti-rabbit conjugated to Alexa 488 (1:500) as previously reported (Qi *et al.* 2008). Sections were cover-slipped using anti-fade medium (Dako, Botany Bay, NSW, Australia). An Olympus microscope (BMX50) equipped with a UV light filter (excitation 330-380nm, barrier 420nm) was used to map the distribution of FG cells, kisspeptin cells and melanocortin cells, including the counting of cells co-localising FG and peptide, using MD plot system (Version 5.0; AccuStage, Shoreview, Minn., USA).

3.3.7 In situ hybridisation

In situ hybridisation (Experiment 3) was performed using 35S dUTP-labelled riboprobes according to a described protocol (Simmons DM 1989; Scott *et al.* 2000). The cDNA and plasmid inserts used were a 375 base ovine Kiss1 gene and a 207 base ovine prepro-ORX gene which were both inserted into a pGem T-easy plasmid. The amplification and linearisation of plasmid DNA was performed using standard techniques (Sambrook *et al.* 1989). When analysing ORX gene expression, 2 anatomically matching sections across the DMH, peri-fornical (PFA), zona incerta (ZI) and the lateral hypothalamic (LHA) areas were chosen for each animal as this is where the majority of ORX expressing cells are located in the ewe (Iqbal *et al.* 2001c). For analysis of Kiss1 expression, 3 sections from each ewe were taken to represent rostral, middle and caudal regions of the ARC and 3-5 sections through the dorso-lateral POA were chosen for analysis of Kiss1 expression in this region. Following hybridisation, the slides were dipped in Ilford K5 photographic emulsion (Ilford Imaging, Melbourne, Australia) and kept at 4°C in the dark and developed after 4 days (ORX), 7 days (Kiss1 ARC), or 9 days (Kiss1 POA). Sections were then

counterstained with 1% cresyl violet, dehydrated and cover-slipped using DPX. Image analysis was carried out using coded slides and the operator was blind to the treatments. Cells were counted when silver grain density was >5 times background and when there was a clearly discernible nucleus. Computer assisted grain counting was performed under bright light field at 40X using a Fuji HC-2000 high resolution digital camera and Analytical Imaging system 3.0 software (Image Pro Plus), as previously described (Henry *et al.* 2001b). The number of silver grains/cell for Kiss1 mRNA was estimated in 40 cells/section in the ARC and 20 cells/section in the POA. ORX expression was analysed in the DMH, PFA, ZI and LHA and silver grains/cell was estimated in 40cells/region.

3.3.8 Radioimmunoassays

Plasma LH was measured by assaying samples in duplicate at 100µl following the method of Lee *et al*. (Lee *et al.* 1976). Ovine standards were NIH-oLH-S18 and ovine antiserum (NIDDK-anti-oLH-I) was used with ^{125}I-NIDDK-AFD-9598B as tracer. The sensitivity of the LH assays was 0.1ng/ml (experiment 1) and 0.2ng/ml (experiment 4). For all LH assays the inter-assay coefficient of variation (CV) less than 15% and the intra-assay CV less than 8%. An LH pulse was defined as having occurred when the assay value of a given sample exceeded the assay value of the previous sample by at least 3 standard deviations, as well as other criteria detailed previously (Clarke and Cummins 1985b).

Plasma progesterone levels were measured in a single assay based on the method of (Deayton *et al.* 1993), after hexane extraction of 100 µl plasma with a sensitivity of 0.1ng/ml and an intra-assay coefficient of variation of 5%.

3.3.9 Statistics

Data are presented as means (±SEM). Hormone data were analysed by repeated measures ANOVA, with Least Significant Differences as a *post-hoc* test. Measures of mRNA levels were analysed by one-way independent measures ANOVA.

3.4 Results

3.4.1 Experiment 1- MTII treatment during the luteal phase of the estrous cycle

Compared to pre-infusion values, MTII treatment increased pulsatile LH secretion (Figure 3.1 A) by increasing mean plasma LH concentration ($p<0.05$) and LH pulse frequency ($p<0.05$), with no effect on LH pulse amplitude (Table 3.1). Infusion of aCSF had no effect on plasma LH levels (Figure 3.1 B). All values obtained below the sensitivity of the LH radioimmunoassay were taken at the sensitivity threshold of 0.09 ng/ml.

	Mean LH (ng/ml)		LH pulse frequency (pulses/h)		LH pulse amplitude (ng/ml)	
	Before	**After**	**Before**	**After**	**Before**	**After**
MTII	0.16 ± 0.016	0.46 ± 0.09*	0.42 ± 0.08	1.08 ± 0.16*	0.73 ± 0.22	0.68 ± 0.03
Vehicle	0.1 0 ± 0.08	0.05 ± 0.04	0.25 ± 0.25	0.17 ± 0.09	0.21 ± 0.21	0.25 ± 0.18

Table 3.1: Analysis of mean LH concentration (ng/ml), LH pulse frequency (pulses/h) and LH pulse amplitude (ng/ml) before and after MTII or aCSF icv infusion to ewes (n=4/group) in the luteal phase of the estrous cycle. Data are means ± SEM. * $p<0.05$ compared to pre-infusion values.

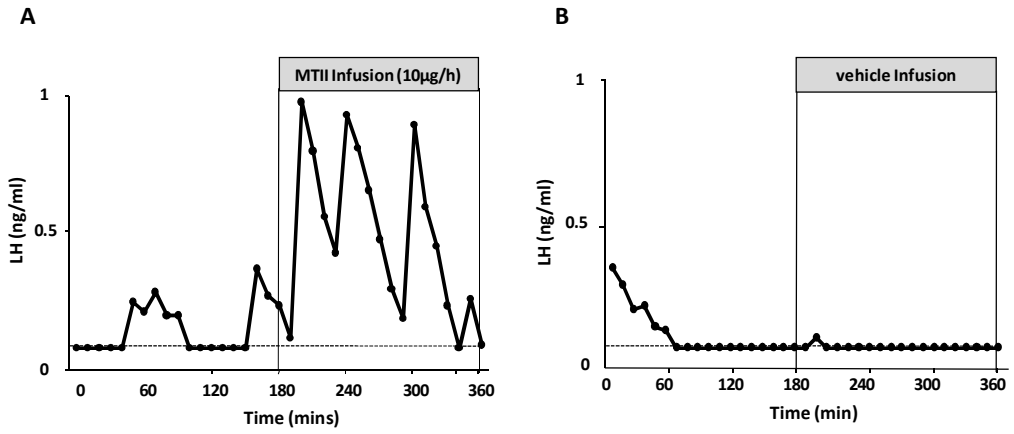

Figure 3.1: Representative profile of the effect of lateral ventricular infusion of a melanocortin agonist, MTII (10µg/h;n=4) **(A)** or aCSF (n=4) **(B)** for three hours on plasma LH levels from ewes in the luteal phase of the estrous cycle. Plasma samples were collected every 10 min for 3h before and after infusion. *Dotted* base line is representative of LH assay sensitivity (0.09ng/ml).

3.4.2 Experiment 2 –Projections of Kisspeptin and Melanocortin cells from the ARC to the POA (retrograde labelling)

All FG injections sites were located in the ventromedial POA and positioned around the third ventricle (Figure 3.2 A,B) where GnRH neurons are located (Figure 3.2 C). Co-localisation of FG cells with POMC and kisspeptin cells in the ARC was rarely seen, so that 1±0.6% of POMC (γ-MSH-immunoreactive) and 1.9±0.2% kisspeptin cells were observed to be labelled with FG.

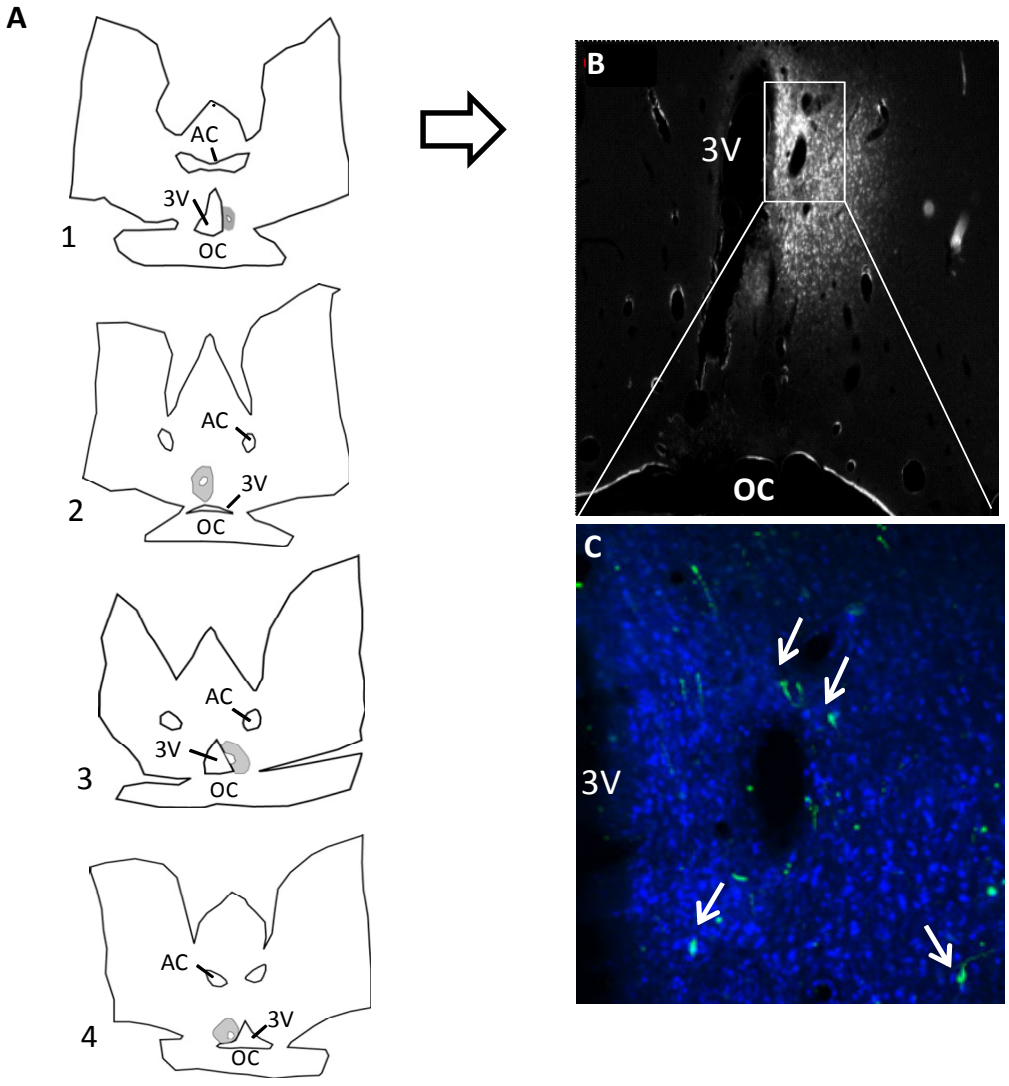

Figure 3.2: (A) Schematic representations of the injection sites in all four animals. Inner white circle indicates injection site, while the grey surrounding shading specifies the extent of the spread of tracer. **(B)** Low power (2.5X magnification) photomicrograph showing an example of the injection site. **(C)** High power (10X magnification) fluorescent photomicrograph showing the injection site (blue) encompassing GnRH cells (green; arrows point to GnRH cell bodies). OC = Optic Chiasm, AC = Anterior Commissure, 3V = Third ventricle.

3.4.3 Experiment 3 - ORX and Kiss1 gene expression following MTII treatment

MTII treatment increased ORX mRNA expression in the DMH (Figure 3.3) by increasing the number of detectable cells ($p<0.05$) and the level of expression (grains per cell, $p<0.01$), but there was no effect of MTII treatment on ORX gene expression in the PFA, ZI or LHA (Figure 3.3 A,B).

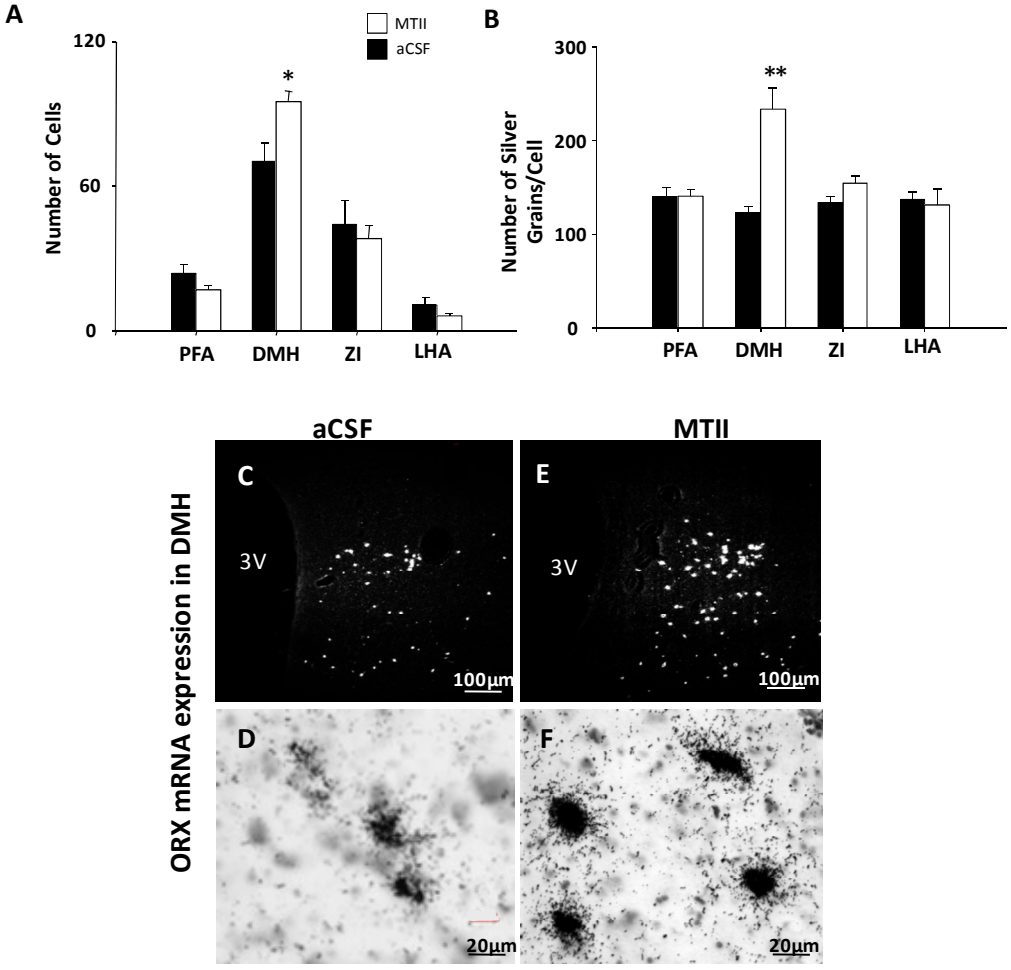

Figure 3.3: Effect of lateral ventricular infusion of aCSF (n=4) or a melanocortin agonist, MTII (10µg/h;n=4) for 20h on ORX gene expression in the perifornical (PFA), dorsomedial hypothalamus (DMH), zona incerta (ZI), and lateral hypothalamus (LHA), expressed by the average number of cells per section and the number of silver grains per cell **(A-B)**. Darkfield (10X) and brightfield (40X) photomicrographs of ORX expression in the DMH **(C-F)**. 3V = Third ventricle. aCSF treatment represented by solid bar; MTII treatment represented by open bar. Data are means ±SEM. *p<0.05 **p<0.01 compared to aCSF treatment.

MTII treatment in the luteal phase reduced ($p<0.05$) Kiss1 mRNA expression in the ARC and increased ($p<0.05$) Kiss1 mRNA expression in the POA. In both cases this was due to a change in the level of expression/cell and not the number of cells detected (Figure 3.4).

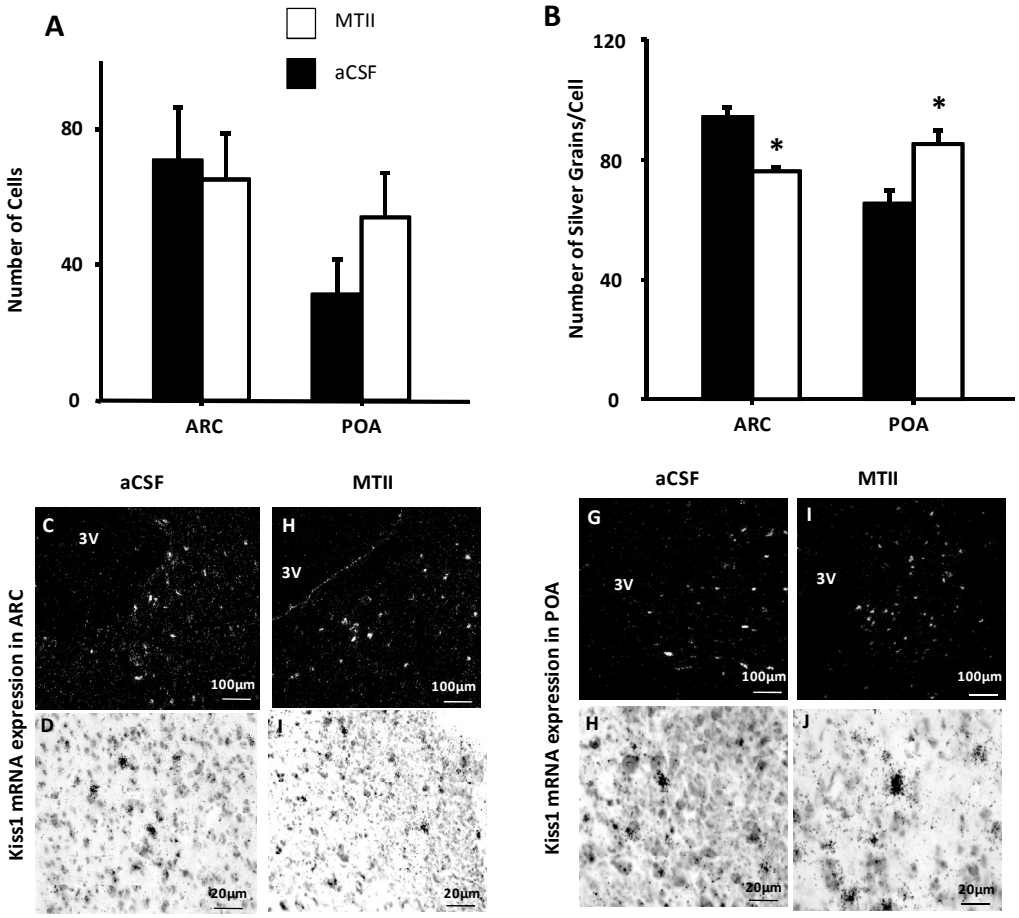

Figure 3.4: Effect of lateral ventricular infusion of aCSF (n=4) or melanocortin agonist, MTII (10µg/h;n=4) for 20h on Kiss1 gene expression in the arcuate nucleus (ARC) and preoptic area (POA) of the ewe, expressed by the average number of cells per section and the number of silver grains per cell **(A-B)**. Darkfield (10X) and brightfield (40X) photomicrograph representations of ARC **(C-F)** and POA **(F-J)**. aCSF treatment represented by solid bar; MTII treatment represented by open bar. Data are means ±SEM. *$p<0.05$ compared to aCSF treatment.

3.4.4 Experiment 4 - MTII treatment of acyclic anestrous ewes

Although plasma LH levels rose immediately after the start of infusion of MTII, from non-detectable to 1.0±0.4ng/ml, this rise was not statistically significant ($p<0.09$). Plasma LH levels were significantly ($p<0.05$) elevated at the 4h time point. No discernable LH surges were detected over the period during which we sampled (Figure 3.5), nor was there any evidence of an increase in mean post-infusion progesterone levels between the two treatment groups (MTII treated: 0.1±0.04ng/ml, Vehicle: 0.3±0.10ng/ml), which would have indicated active corpora lutea following ovulation. All values obtained below the sensitivity of the LH radioimmunoassay were taken at the sensitivity threshold of 0.2ng/ml.

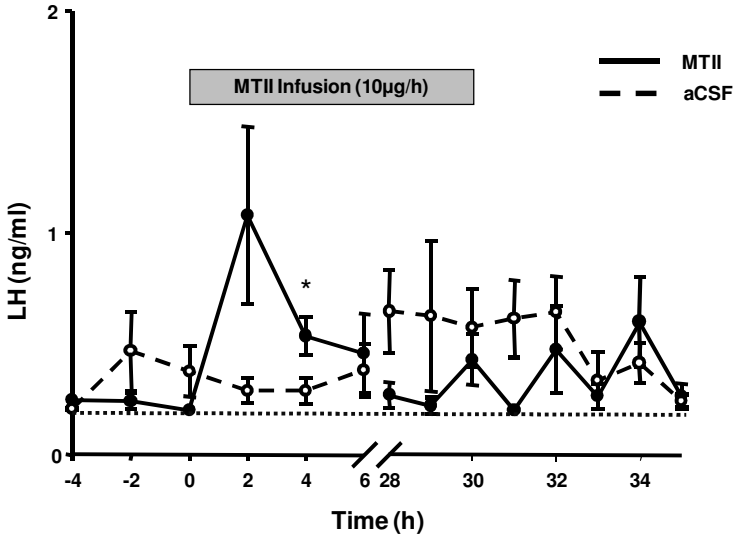

Figure 3.5: Effect of lateral ventricular infusion of aCSF (n=6) or melanocortin agonist, MTII (10µg/h; n=6) for 30h on LH secretion in anestrous ewes. Blood samples were collected every 2h. *Continuous* line represents MTII treated ewes and *dashed* line represents aCSF treated ewes. *Dotted* base line is representative of LH assay sensitivity (0.2ng/ml). Data are means ±SEM. *p<0.05 compared to aCSF LH concentration.

3.5 Discussion

These studies substantiate a growing body of evidence to indicate that the melanocortin system within the brain acts to stimulate the reproductive axis. The present results support other recent data obtained in lean hypogonadotropic ewes to suggest that the means by which leptin activates the GnRH/gonadotropin axis is through the melanocortin system (Backholer *et al.* In Press-a). During the luteal phase, gonadotropin levels are suppressed due to progesterone/estrogen negative feedback, but melanocortin treatment can overcome this negative clamp. The present study corroborates earlier data (Pompolo *et al.* 2001) which showed that there is relatively poor direct input to the ventromedial POA from the arcuate nucleus. We expanded this earlier work to show that the melanocortin and kisspeptin cells of the ARC do not provide substantial input to the ventromedial POA. There may, however, be an indirect pathway to GnRH cells from the cells of the ARC (Pompolo *et al.* 2001). Expression of ORX mRNA in cells of the DMH and expression of Kiss1 mRNA in cells of the dorso-lateral POA was increased following melanocortin treatment to luteal phase ewes, so these cells may act as intermediary neurons to transmit melanocortin signals to the GnRH neurons.

We have shown that MTII treatment can overcome the sex steroid negative feedback effect on GnRH secretion (Clarke *et al.* 1987) during the luteal phase of the ewe. Earlier reports showed that POMC mRNA in the ARC of the ewe brain (Walsh *et al.* 1998) and total α-MSH protein levels in the mediobasal hypothalamus and POA of the rat brain (Khorram *et al.* 1985) remains unchanged across the estrous cycle. These studies, however, did not take into account the marked differences in the biological properties of the different forms of α-MSH. Acetylated α-MSH is much more potent the des-acetyl form (Tsujii and Bray 1989; Abbott *et al.* 2000) and levels of the former are reduced in conditions of low gonadotropin production in lean animals (Backholer *et al.* In Press-a). Importantly, this recent work showed that the major change in melanocortin status that occurs with altered reproductive state is the level of α-MSH in the terminal beds of melanocortin neurons and not in the ARC. In order to gain a more comprehensive understanding of the operation of the melanocortin system across the estrous

cycle, further investigation of the type of melanocortin that prevails at each stage is required. Plasma levels of acetylated α-MSH in humans are at highest concentration during the late follicular phase, when gonadotropin secretion is correspondingly high (Mauri *et al.* 1990). The present result obtained with MTII treatment of ewes in the luteal phase of the estrous cycle, indicates that melanocortin signalling may be suppressed at this time. This may be due to an effect on the production of acetylated α-MSH, but there may also be effects on production of β- and/or γ–MSH, which also activate the MC3R and MC4R (Gantz *et al.* 1993; Harrold *et al.* 2003). Appropriate proteomic analysis is required to determine whether there is any change in the levels and forms of melanocortins in terminal beds in relevant regions of the hypothalamus and POA across the estrous cycle.

We measured ORX gene expression in animals treated with MTII because of earlier studies showing that approximately one-third of GnRH cells in the ovine brain appear to receive direct input from ORX cells in the lateral hypothalamus (Iqbal *et al.* 2001c). ORX mRNA was up regulated in the DMH following melanocortin treatment of luteal phase ewes. Since this treatment also caused increased plasma LH levels, the data are consistent with earlier work in steroid primed OVX rats showing an increase in LH secretion following ORX treatment (Pu *et al.* 1998). Our earlier anatomical study, however, showed that ORX cells of the DMH do not project to the ventromedial POA in the sheep (Iqbal *et al.* 2001c). To reconcile these findings, and to invoke a role for these ORX cells in the melanocortin stimulation of the reproductive axis, it is necessary to consider the possibility of serial connection to GnRH cells. It is possible that the ORX cells of the DMH project to some other rostrally located area to relay information to GnRH neurons. This pathway may involve interneurons in the BnST and/or the median preoptic nucleus/lateral preoptic area (Pompolo *et al.* 2005). It is also possible that these ORX cells in the DMH act via the kisspeptin cells located in the dorsal-lateral POA (Franceschini *et al.* 2006). Support for the notion that the ORX cells of the DMH form at least part of the response to a melanocortin agonist, that is relayed to GnRH cells is the fact that this nucleus is highly innervated by α-MSH-containing axons and axon terminals originating from the ARC in the rat (Jacobowitz and

O'Donohue 1978) and ablation of the ARC results in a discernible reduction of α-MSH fibres in the DMH (Homma *et al.* 2006). Finally, melanocortin receptors (MC3R and MC4R) are found in the DMH in the ewe (Iqbal *et al.* 2001a). An alternative rationale for the increase in ORX gene expression with MTII treatment may be related to 'appetite' regulation, although this would seem contradictory as ORX stimulates food intake (Sartin *et al.* 2001) and melanocortins (Fan *et al.* 1997) have the reverse effect.

In sheep in the luteal phase of the cycle, MTII treatment reduced Kiss1 mRNA levels in the ARC but increased expression in the POA. Sex steroids, particularly estradiol-17β, negatively regulate Kiss1 mRNA expression in the ARC (Smith *et al.* 2007), and up regulate Kiss1 mRNA expression in the POA (Smith *et al.* 2008a), leading to the perceived role of kisspeptin in facilitating both negative and positive feedback responses of sex steroids to GnRH neurons (Smith 2009). It is important, therefore, to take account of the likely rise in estradiol-17βlevels which result from the increase in LH secretion following melanocortin treatment. It is unknown whether the increase in Kiss1 mRNA in the POA and the decrease in the ARC is a direct effect of melanocortin treatment, or is a consequence of increased estradiol-17β levels. It has been suggested that the MC4R is the principal melanocortin receptor involved in stimulating the reproductive axis (Watanobe *et al.* 1999a), and this subtype is expressed at a high level in the POA, but not in the ARC of the ewe (Iqbal *et al.* 2001a). Furthermore, virtually all of the kisspeptin cells of the ARC express estrogen receptor-α, but only 50% of the same cell type in the POA express the receptor (Franceschini *et al.* 2006). This leads us to tentatively conclude that melanocortins activate the kisspeptin cells of the POA directly, but the decrease in Kiss1 expression seen in the ARC may be due to a rise in sex steroid levels.

MTII treatment caused a small increase in LH levels in anestrous ewes, but this did not translate into an LH surge, ovulation or the formation of corpora lutea. In this experiment, the major objective was to determine whether there was a positive feedback response to the treatment, causing ovulation. Plasma LH levels in samples collected prior to and shortly after commencement of treatment suggest that MTII treatment can at least partly overcome the enhanced negative feedback effect of

estrogen that prevails during anestrus (Legan *et al.* 1977). A more intense series of blood samples around this treatment may provide more detailed data on this point, but it is clear from the present results that MTII treatment cannot reverse the acyclic condition of anestrus. The relatively short-lived response to MTII in these animals may have been due to stimulation of ovarian function, sufficient to increase estradiol-17β secretion and affect a negative feedback response on GnRH/LH secretion, thus preventing sustained activation of the reproductive axis and ovulation. Preovulatory-like LH surges did not occur in any of the treated ewes, nor was there any evidence of ovulation. It is possible that there is reduced responsiveness to melanocortins in the anestrous season. POMC mRNA is increased in anestrous ewes (Clarke *et al.* 2000) and rams (Anukulkitch *et al.* 2007), but it remains to be determined whether MC3R and MC4R expression is changes with season in the ewe.

The present study confirms previous observations with anterograde tracing which indicated relatively poor direct input to the ventromedial POA from the ARC (Pompolo *et al.* 2001). We found that very few POMC or kisspeptin cells were retrogradely labelled when FluoroGold was placed in the ventral POA. Our earlier results (Pompolo *et al.* 2001) were contradicted by those of another group, using relatively large injections of retrograde tracer placed in various regions of the POA and diagonal band of Broca (Goubillon *et al.* 2002), but our present results confirm our earlier findings. This suggests that the observed input to GnRH cells from kisspeptin elements in the ovine brain arises from the kisspeptin cells in the dorsal-lateral POA and not the ARC. Stimulation of the GnRH cells from any elements in the ARC, especially kisspeptin and/or melanocortins, is likely to be indirect. Although only few projections were noted from the ARC kisspeptin and POMC cells to the ventromedial POA where GnRH cells are found, it does not preclude the projection of these cells to the dorso-lateral POA to activate kisspeptin neurons. In support of this notion, Kiss1 mRNA was up regulated in the POA following melanocortin treatment in the luteal phase. The functional significance of the kisspeptin cells in the POA still remains to be fully elucidated in this species, but it has been proposed that these may act in a similar manner to the kisspeptin cells in the AVPV of the rodent, which positively regulate GnRH/LH secretion during the

pre-ovulatory LH surge (Smith 2008). In this regard, it is notable that Kiss1 mRNA in the POA is up regulated in the peri-ovulatory phase in monkeys and in the late follicular phase in sheep (Smith, JT and Clarke IJ, unpublished observations). It is plausible therefore, that the kisspeptin cells of the POA are involved in the positive regulation of GnRH neurons and act as an inter-neuronal pathway for the kisspeptin and/or melanocortin cells of the ARC. Alternatively, GnRH secretion may be regulated by melanocortins and/or kisspeptin cells at the level of the median eminence. Recent studies suggest that there is some association between kisspeptin fibres and GnRH fibres in the median eminence (Ramaswamy *et al.* 2008), and several reports demonstrate that POMC cells project to the median eminence (Conover *et al.* 1993). On the other hand, there is no substantive evidence for synaptic input to GnRH axons and terminals within the median eminence and communication may be by volume transmission (Kawakami *et al.* 1998; Durrant and Plant 1999; Yin *et al.* 2007).

In conclusion, this study adds further impetus to the notion that the melanocortins are of fundamental importance to the normal reproductive process. We have reinforced the fact that melanocortin signalling positively regulates GnRH cells, since MTII has a convincing effect to increase pulsatile LH secretion in luteal phase ewes and is likely to increase basal LH levels during seasonal anestrous. Evidence is presented that this effect may be transmitted via ORX and/or kisspeptin cells in the DMH and the POA respectively. Understanding the involvement of the melanocortin system in stimulating the reproductive axis builds evidence to support the use of melanocortins as a therapeutic target in times of reproductive suppression.

3.6 Acknowledgments

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Chapter 4

Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells to stimulate reproduction

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This manuscript will be submitted to 'Journal of Neuroscience'

Declaration for Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiment management/conduct, laboratory analysis, data collection, statistical analysis, preparation of manuscript	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Iain J Clarke[1]	Provided intellectual input and editing of manuscript	N/A
Jeremy T Smith[1]	Provided intellectual input and editing of manuscript	N/A
Javed Iqbal[1]	Experiment conduct	N/A
Alda Pereira[1]	Experiment conduct	N/A
Alix Rao[1]	Experiment conduct	N/A

Candidate's Signature		Date

Declaration by co-authors

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(16) there are no other authors of the publication according to these criteria;
(17) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(18) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	[1]Monash University, Department of Physiology, Clayton Campus

		Date
Signature 1		
Signature 2		
Signature 3		
Signature 4		
Signature 5		

Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells to stimulate reproduction

4.1 Abstract

Kisspeptin stimulates reproduction and cells in the arcuate nucleus (ARC) that produce kisspeptin express Ob-Rb in the mouse. Here, we report studies in ewes to determine 1) whether kisspeptin cells respond to leptin and express Ob-Rb and 2) if reciprocal connections exist between kisspeptin cells and pro-opiomelanocortin (POMC) and neuropeptide Y (NPY) cells to modulate reproductive and metabolic homeostasis. Kiss1 mRNA was measured by *in situ* hybridisation in ovariectomised (OVX) ewes that were 1) normal body weight, 2) lean or 3) lean with leptin treatment by intracerebroventricular (icv) infusion (4µg/h, 3 days). Kiss1 mRNA expression in the ARC and the preoptic area (POA) was lower in hypogonadotropic lean animals than in those of normal weight, and intracerebroventricular (icv) infusion of leptin partially restored Kiss1 mRNA expression in lean animals. Single cell laser capture microdissection coupled with real-time PCR showed that kisspeptin cells (POA and ARC) express Ob-Rb. Double-label fluorescent immunohistochemistry showed that reciprocal connections exist between kisspeptin cells and NPY and POMC cells. Accordingly, we treated OVX ewes with kisspeptin (5µg/h, icv) or vehicle for 20h and examined POMC and NPY gene expression by *in situ* hybridisation. Kisspeptin treatment reduced POMC and increased NPY gene expression. Thus, kisspeptin neurons respond to leptin and expression of Kiss1 mRNA is affected by leptin status. Kisspeptin cells communicate with NPY and POMC cells, altering expression of the relevant genes in the target cells; reciprocal connections also exist. These data provide a network between the 3 cell types that could co-ordinate control of reproduction and metabolic homeostatic systems.

4.2 Introduction

There is now unequivocal evidence that the kisspeptin system stimulates the reproductive axis in various species including rodents (Irwig *et al.* 2004), sheep (Smith *et al.* 2006b), monkeys (Shahab *et al.* 2005), and humans (Dhillo *et al.* 2005). There is also evidence that the kisspeptin cells of the brain respond to metabolic signals and may relay relevant information to GnRH cells, since Kiss1 mRNA is reduced in both pre-pubertal (Castellano *et al.* 2005) and adult (Luque *et al.* 2007; Brown *et al.* 2008) male and female rats deprived of food. Kisspeptin cells in the arcuate nucleus (ARC) express the signalling form of the leptin receptor (Ob-Rb) (Smith *et al.* 2006a) and leptin treatment increases Kiss1 mRNA when administered to the N6 murine hypothalamic mouse cell line (Luque *et al.* 2007). Furthermore, repeated administration of kisspeptin to uncontrolled diabetic rats restores the hypogonadotropic axis (Castellano *et al.* 2006). Reproductive function can also be restored in hypogonadal lean rats (Castellano *et al.* 2005) and *ob/ob* mice of low fertility (Barash *et al.* 1996; Chehab *et al.* 1996) by central and intraperitoneal administration of kisspeptin. Kiss1 mRNA is decreased in leptin deficient *ob/ob* mice and leptin treatment stimulates Kiss1 mRNA expression (Smith *et al.* 2006a). In lean hypogonadotropic ovariectomised (OVX) ewes, leptin restores pulsatile LH secretion (Henry *et al.* 2001a). The effect of lean condition and the effect of leptin on Kiss1 mRNA has not been studied in this animal model.

The regulatory role of leptin on the kisspeptin system in rodent species suggests a means by which the reproductive axis can be 'informed' of metabolic status, but the central circuits that are involved are not elucidated. Many peptides within the brain that are involved in energy balance also act to regulate gonadotropin releasing hormone (GnRH) secretion (Crown *et al.* 2007), such as Neuropeptide Y (NPY) and melanocortins derived from the POMC gene (Nijenhuis *et al.* 2001; Schioth *et al.* 2001; Clarke *et al.* 2005; Cone 2005; Crown *et al.* 2007). NPY stimulates food intake and inhibits reproduction in the sheep (Clarke *et al.* 2005), whereas the melanocortins reduce food intake (Fan *et al.* 1997) and stimulate reproduction in this species (Watanobe *et al.* 1999a). Leptin acts to down regulate NPY expression in animals of normal body weight (Wang *et al.* 1997; Henry *et al.*

1999; Ahima and Hileman 2000), but does not do so in lean animals (Backholer *et al.* In Press-a). Altering body weight has been observed to have variable effects on POMC gene expression (Henry *et al.* 2000; Korner *et al.* 2001). In recent studies, however, we found that POMC gene expression was reduced in lean, hypogonadotropic OVX ewes and restoration of expression to normal levels could be effected by leptin treatment (Backholer *et al.* In Press-a). Similarly, leptin positively regulates POMC mRNA expression in rodent species (Thornton *et al.* 1997; Ahima and Hileman 2000). On this basis, we hypothesised that melanocortins positively regulate kisspeptin cells. Regarding NPY, a decrease in Kiss1 gene expression was seen in NPY knockout mice and NPY stimulated Kiss1 gene expression in hypothalamic N6 cell lines (Luque *et al.* 2007), suggesting that NPY may positively stimulate kisspeptin cells. There are no reports implicating the kisspeptin system in the regulation of POMC and/or NPY expression, nor do data exist regarding the link between metabolic regulators and the kisspeptin system in the sheep.

The aim of the present study was to further interrogate the role of the kisspeptin system in relaying metabolic information to the reproductive axis in the ewe. We tested the hypothesis that the kisspeptin system responds to leptin status by measuring Kiss1 mRNA in lean hypogonadotropic animals with and without leptin treatment and in normal weight animals. Because Kiss1 mRNA was reduced in lean animals and leptin was able to partially restore Kiss1 mRNA expression we then investigated the possibility that the kisspeptin system communicates with other neuronal systems involved in metabolic regulation and the reproductive axis, *viz.* the NPY and POMC systems of the ARC. Our results show that POMC and NPY cells receive neuronal input from kisspeptin-immunoreactive cells of the ARC, and that POMC gene expression is reduced and NPY gene expression is increased following kisspeptin treatment of ovariectomised (OVX) ewes. Furthermore, we demonstrate that reciprocal communication exists between these cells types. We conclude that the kisspeptin cells of the ovine ARC and preoptic area (POA) respond to leptin and a mutual connection exists between the ARC kisspeptin and POMC/NPY cells. Thus, kisspeptin cells may have dual function to regulate both reproduction and metabolic homeostasis.

4.3 Materials and Methods

4.3.1 Animals

Adult Corriedale ewes were maintained on pasture or in feedlots. For experimentation, the animals were housed in individual pens with natural lighting and had access to water *ad libitum.* The animals were conditioned to pen-housing and handling for 1 week prior to experimentation and third ventricular (IIIV) cannulation was carried out at least 2 weeks before experimentation as previously described (Barker-Gibb *et al.* 1995). All animals were ovariectomised (OVX) at least one month beforehand, to eliminate cyclic alterations in the secretion of gonadal steroids. Lean animals were subjected to dietary restriction over a period of 6-10 months as described previously (Henry *et al.* 2000). Briefly, they were fed 500g of pasture hay/day supplemented with straw for bulk, to achieve body condition scores of 2 (Russel 1969). Animals of normal body weight were kept on pasture, with hay supplementation for maintenance. The animals were weighed monthly and adjustments in food intake were made, so that target weights of approximately 35kg (lean) and 55kg (normal) were attained. No animal in the lean group was excluded from feeding by dominant flock-mates. When in single pens, lean animals were fed 500g of lucerne chaff/day and normal animals had *ad libitum* access to food. All animal procedures were conducted with prior institutional ethical approval of the Animal Experimentation Ethics Committee of Monash University fulfilling the requirements of the Australian Prevention of Cruelty to Animals Act 1986 and the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Australian Animal Commission *Code of Practice for the Care and Use of Animals for Scientific Purposes*.

4.3.2 Experiment 1 - Effect of body weight and leptin treatment on the Kiss1 gene expression.

Lean (33 ± 2.9 kg) OVX ewes (5/group) received IIIV infusions of either 4µg/h human recombinant leptin (Henry *et al.* 2001a) or artificial cerebrospinal fluid (aCSF; 150mM NaCl, 1.2mMCaCl, 1mM MgCl and 2.8 mM KCl) and normal (n=4) weight ewes received aCSF as vehicle at a rate of 55µl/h for 3 days using MS16A

Grasby mini-pumps (Graseby Medical Ltd., Gold Coast, Australia). Blood samples (5 ml) were collected every 10mins for 6h before infusion and for the final 6h of the 72h infusion period. Plasma was harvested and stored at -20°C until assayed for LH. At the end of the infusion period, animals were euthanised by overdose of 20 ml sodium pentobarbital (Lethabarb; Virbac, Peakhurst, NSW, Australia) iv and the heads were removed and perfused through both carotid arteries with 2L normal saline containing heparin (12.5U/ml) followed by 3L of 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4, the final litre containing 20% sucrose. Brains were removed, and the hypothalamus and POA was dissected as blocks and post-fixed at 4°C in fixative containing 30% sucrose for 7 days. Cryostat sections were cut in the coronal plane (20μm), collected into cryoprotectant with 2% paraformaldehyde and stored at -20°C until Kiss1 mRNA expression was quantified by *in situ* hybridisation (*vide infra*). These animals had been used in a previous experiment (Backholer *et al.* In Press-a).

4.3.3 Experiment 2 – Expression of Ob-Rb in Kiss1 cells of the ARC and POA

Since leptin was found to regulate Kiss1 mRNA expression in both the ARC and the POA we determined whether these cells express the signalling leptin receptor, Ob-Rb. The brains of 2 OVX ewes of normal weight were perfused as above and the hypothalami and POA were dissected as blocks and stored in RNAse free 0.1M PBS until cryostat sections were cut in the coronal plane (20μm) and collected into cryoprotectant. The sections were stored at -20°C, prior to immunohistochemical staining, laser capture and real time PCR (*vide infra*).

4.3.4 Experiment 3 –Reciprocal connections between kisspeptin cells and POMC/NPY cells in the ARC

OVX and gonad-intact ewes of normal body weight (n=4/group) were euthanised and the brains were perfused as above. Cryostat sections were cut in the coronal plane (40um), collected into cryoprotectant and stored at -20°C for later immunohistochemical analysis of the connectivity between kisspeptin cells and the POMC and NPY cells of the ARC (*vide infra*).

4.3.5 Experiment 4 – Kisspeptin regulates POMC and NPY gene expression

OVX ewes (4/group) received IIIV infusion of 5µg/h of kisspeptin (Phoenix Pharmaceuticals, CA) or aCSF at an infusion rate of 100µl/h for 20h after being fitted with icv infusion cannulae (SILASTIC brand tubing, ID 1.02 mm, outer diameter 2.16 mm) in the lateral ventricle (Henry *et al.* 2008) connected to 5-ml plastic syringes driven by Graseby MS16A infusion pumps (Smith Medical Australasia Pty. Ltd., Gold Coast, Queensland, Australia). Blood samples were collected every half hour for 1h before and after the commencement of the infusion to monitor the LH response to kisspeptin. Plasma was harvested and stored at -20°C until assayed for an acute LH response. At the end of the 20h infusion period, animals were euthanised and brains were perfused and sectioned as above to measure POMC and NPY gene expression by *in situ* hybridisation (*vide infra*).

4.3.6 In situ hybridisation

This was performed using ^{35}S dUTP-labelled riboprobes according to a described protocol (Simmons DM 1989; Scott *et al.* 2000). The cDNA and plasmid inserts used were a 375 base ovine Kiss1 sequence (Smith *et al.* 2007), a 400 base ovine POMC sequence (van de Pavert *et al.* 1997) and a 511 base rat NPY sequence donated by Dr Steven Sabol (National Heart, Blood and Lung Institute, Bethesda, MD, USA) (Estrada *et al.* 2003b). The amplification and linearisation of plasmid DNA was performed using standard techniques (Sambrook *et al.* 1989). For analysis of ARC Kiss1, POMC and NPY expression, 3 sections from each ewe were taken to represent rostral, middle and caudal regions of the ARC. Three to five sections through the POA were chosen for Kiss1 mRNA expression analysis in the dorsolateral region. All cRNA probes were synthesised using a Promega Gemini System II kit (Promega, Annandale, NSW, Australia). Following hybridisation, the slides were dipped in Ilford K5 photographic emulsion (Ilford Imaging, Melbourne, Australia) and kept at 4°C in the dark and developed after 9 days (Kiss1 in ARC), 12 days (Kiss1 POA), 7 days (POMC) or 11 days (NPY). Sections were then counterstained with 1% cresyl violet, dehydrated and coverslipped using DPX. Image analysis was carried out using coded slides and the operator was blind to the

treatments. Cells were counted when silver grain density was >5 times background and when there was a clearly discernible nucleus. Computer assisted grain counting was performed under bright light field at 40X using a Fuji HC-2000 high resolution digital camera and Analytical Imaging system 3.0 software (Image Pro Plus), as previously described (Henry *et al.* 2001b). The number of silver grains/cell for ARC Kiss1, POMC and NPY mRNA was estimated in 40 cells per section in the ARC. In the POA Kiss1 mRNA silver grains/cell was estimated in 20 cells per section or all cells if less than 20 cells were visible on the section.

4.3.7 Laser capture microdissection and real-time PCR for the determination of Ob-Rb on Kisspeptin cells of the ARC and POA

Mid-ARC and POA sections were selected from the tissues of sheep (n=2) in experiment 2 and kisspeptin cells were identified in free floating sections by RNAase free immunohistochemistry. Briefly, sections were first washed in 0.1M PBS and pre-incubated in proteinase K digestion for 5 mins at 37°C. After washing sections again in 0.1M PBS, sections were blocked (0.1M PBS/1%BSA/0.3% Triton X100) and kisspeptin cells were identified using a polyclonal rabbit antibody against mouse kisspeptin-10 (gift from Dr A. Caraty, INRA, France; Ref (Franceschini *et al.* 2006) at a dilution of 1:2000 in 0.1M PBS/1% BSA/0.3% Triton X100/0.1% NaN_3 at 24h at room temperature. This antibody has been validated for use in ovine brain tissues (Goodman *et al.* 2007). Sections were then rinsed in 0.1M PBS and incubated for 30 mins in Goat anti-rabbit Alexa 488 secondary antibody (1:500, Molecular Probes, Inc, Eugene, OR) diluted in 0.1M PBS. After a final wash, the sections were mounted onto membrane slides and allowed to dry overnight. Single cells were then isolated using the PALM® MicroLaser Microdissection System (P.A.L.M. MicroLaser Technologies AG, Burnried, Germany). Kisspeptin cells (50 ARC, 20 POA and 2 ARC single cell) were dissected off the slide with a single defocused laser pulse and catapulted directly into the cap of microfuge tubes containing 40µl of RLT buffer from RNeasy Micro Kit (QIAGEN, Hilden, Germany). Total RNA was extracted from the laser captured cells using RNeasy Micro Kit (QIAGEN, Hilden, Germany) and reverse transcribed using AffinityScript QPCR cDNA Synthesis Kit (STRATAGENE, La Jolla, CA). Nested primers were used to enhance transcript detection and measurement. The initial PCR

reaction using outer primers and one tenth of the laser captured cell RT cDNA was set up for 95°C for 5 min, 15 cycles at 95°C for 1 min, 50°C for 1.30 min, 72°C for 1 min, and one cycle at 72°C for 5 min in a 50ul reaction volume using GoTaq® DNA Polymerase (Promega, Madison, WI). Semi-quantitative real-time PCR was performed on the Eppendorf Realplex$_{4.}$ PCR (Eppendorf, Hamburg, Germany) machine using a reaction volume of 20ul containing the inner primers and 2ul of the first round PCR reaction. The PCR conditions were 95°C for 10 min followed by 50 cycles at 95°C for 15 sec, 56°C for 1 min and 72°C for 30 sec using Brilliant® SYBR® Green QRT-PCR Master Mix (STRATAGENE, La Jolla, CA). For each gene of interest, purified DNA of known concentration was used as the assay standard. In the initial optimisation of each primer set, PCR products were separated by agarose gel electrophoresis, purified and sequenced to confirm their identity. The estimated mRNA concentrations were determined relative to the standard preparation (concentration determined by Nanophotometer) using the Realplex$_4$ computer software. Leptin receptor mRNA expression was corrected with reference gene Malate Dehydrogenase.

4.3.8 Immunohistochemistry

Anatomically matching sections representing rostral, middle and caudal ARC were selected from OVX and ovary intact ewes (n=4/group) of experiment 3 and were mounted onto superfrost slides and dried overnight. Antigen retrieval was performed using 1 M citrate buffer (pH 6) in a microwave oven at 1000 W (2 x 5 min). A blocking solution containing 10% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS was applied, and then sections were incubated for 72 h at 4 C with primary antibody. POMC containing cells were identified using a γ-MSH antibody (Antibodies Australia, Melbourne) with specificity as previously described (Goodman *et al.* 2007). Pre-absorption with 0.5mg/ml of the original peptide abolished all staining in the ovine ARC (data not shown). To detect NPY a monoclonal mouse antibody against NPY (1:4000, 72h at 4°C; courtesy of E. Grouzmann, University hospital, Lausanne, Switzerland) was used. Anti-guinea pig and anti-mouse secondary antibodies conjugated to Alexa 488 (1:500, Molecular Probes, Inc, Eugene, OR) were used as detection methods for γ-MSH and NPY

respectively for 2 hours at room temperature. Following staining for γ-MSH or NPY, the sections were then labelled for kisspeptin. To detect kisspeptin, we used a polyclonal rabbit antibody against mouse kisspeptin-10 (dilution 1:100,000; gift from A. Caraty, INRA, Nouzilly, France) (Franceschini *et al.* 2006) previously validated for use in sheep tissues (Goodman *et al.* 2007). Secondary antibody for kisspeptin staining was anti-rabbit conjugated to Alexa 546 (1:500, Molecular Probes, Inc, Eugene, OR). Sections were finally counterstained with 0.3% Sudan Black B and coverslipped using anti-fade medium (Dako, Botany Bay, NSW, Australia). Putative contacts of kisspeptin fibres on NPY and POMC neurons, and the reciprocal connections, were examined with a Zeiss Apotome microscope (Carl Zeiss, Inc. North Ryde, Sydney, Australia). Z-stacks of optical sections (1 μm; x126 magnification) were captured through kisspeptin, NPY, and POMC immuno-reactive neurons. Putative contacts were defined as apposition of terminals with soma or proximal dendrites when there was no pixilation between the two objects. Using the Apotome system, Z-stacks were rotated to confirm the lack of pixilation between the objects, when viewed in different planes. This method has been reported previously (Qi *et al.* 2009b).

4.3.9 Radioimmunoassays

Plasma LH was measured by assaying samples in duplicate at 100μl following the method of Lee *et al*. (Lee *et al.* 1976). Ovine standards were NIH-oLH-S18 and ovine antiserum (NIDDK-anti-oLH-I) was used with ^{125}I-NIDDK-AFD-9598B as tracer. The average sensitivity of the assays was 0.2ng/ml and the inter-assay coefficient of variation (CV) was less than 15%. The intra-assay CV was less than 9%.

4.3.10 Statistics

Data are presented as means (±SEM). Hormone data were analysed by repeated measures ANOVA, with Least Significant Differences (LSD) as a *post-hoc* test. Measures of mRNA levels and close appositions were analysed by one-way independent measures ANOVA, with LSD *post-hoc* comparison of normal weight, lean, and lean + leptin treated animals. The percentage of cells with close appositions was examined using arc-sine transformed data.

4.4 Results

4.4.1 Experiment 1 - Effect of leptin on Kiss1 mRNA expression

The plasma LH responses to leptin or vehicle in these animals has been reported previously (Backholer *et al.* In Press-a). In summary, vehicle infusion had no effect on mean plasma LH levels, but leptin infusion increased mean LH concentrations ($p<0.01$), LH pulse amplitude ($p<0.01$) and LH pre-pulse nadir ($p<0.01$) in lean hypogonadotropic OVX ewes.

Kiss1 mRNA expression in the ARC was significantly down regulated in lean hypogonadotropic OVX ewes ($p<0.01$; cell number and silver grains/cell), as compared to expression in ewes of normal weight. Leptin treatment partially restored Kiss1 mRNA towards normal by increasing the level of expression (silver grains/cell) ($p<0.05$) (Figure 4.1).

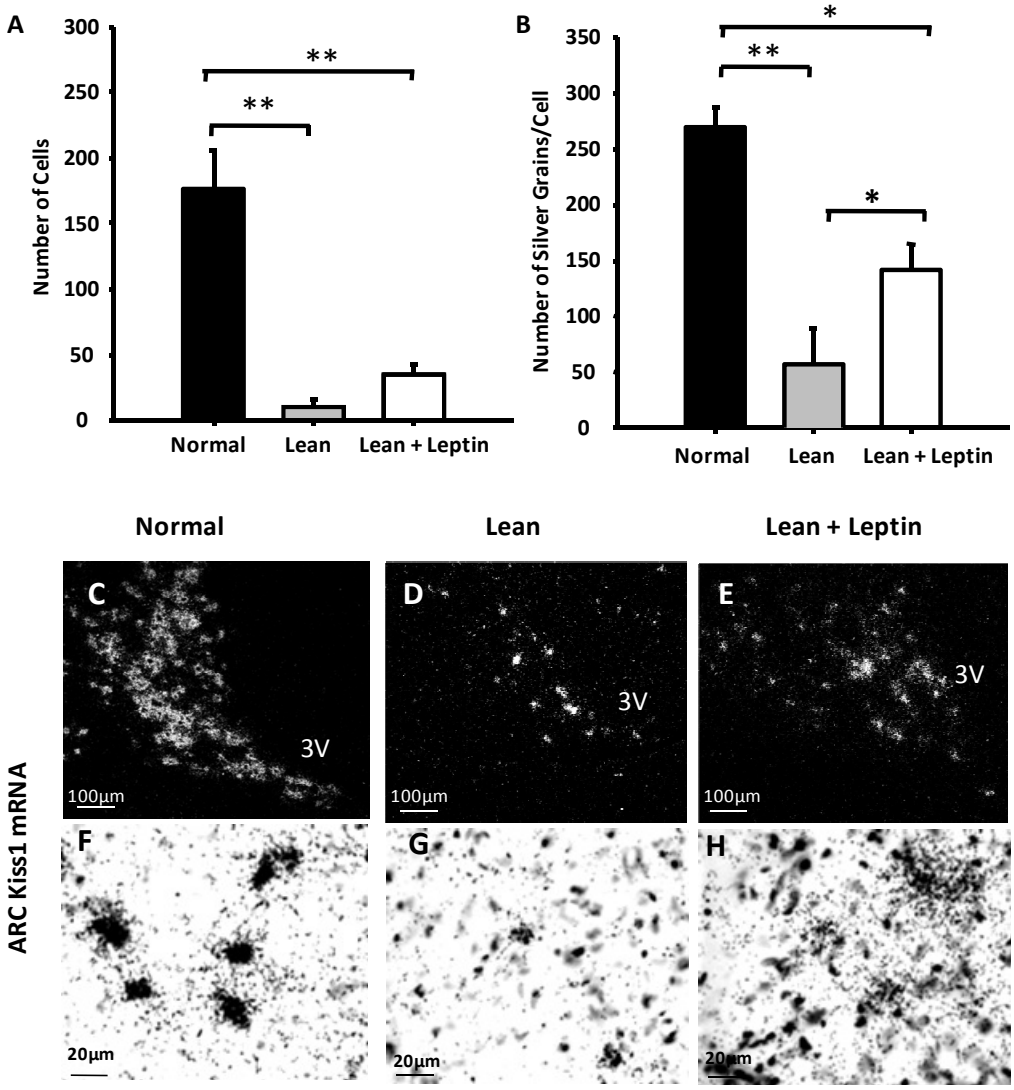

Figure 4.1: Kiss1 mRNA expression in the ARC of normal weight, lean and lean + leptin treated OVX ewes, as determined by *in situ* hybridisation expressed as number of detectable kisspeptin cells **(A)** or silver grains per cell **(B)**. Darkfield photomicrographs of representative ARC sections from normal weight **(C)**, lean **(D)** and lean + leptin treated **(E)** OVX ewes at 10X magnification. Brightfield photomicrographs of representative kisspeptin cells in the ARC of normal weight **(F)**, lean **(G)** and lean + leptin treated **(H)** OVX ewes at 40X magnification. Data are means ± SEM. *p<0.05, **p<0.01

Kiss1 mRNA expression in the POA was significantly down-regulated in lean hypogonadotropic OVX ewes, in terms of the number of detectable cells ($p<0.05$) and the level of expression/cell ($p<0.01$). Leptin treatment to lean hypogonadotropic OVX ewes partially restored the level of Kiss1 mRNA expression/cell ($p<0.05$) but not the number of detectable cells (Figure 4.2).

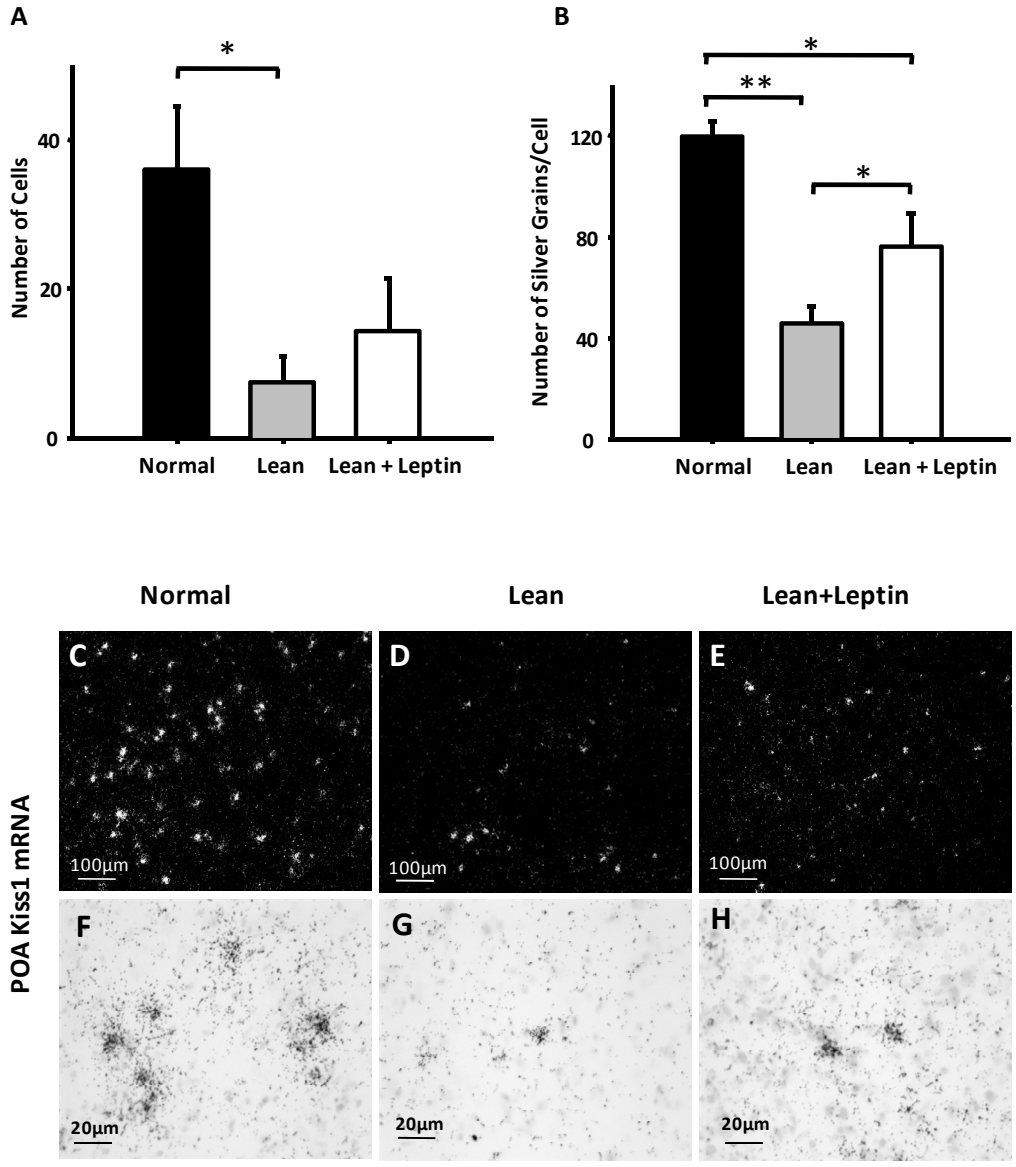

Figure 4.2: Kiss1 mRNA expression in the POA of normal weight, lean and lean + leptin treated OVX ewes, as determined by *in situ* hybridisation expressed as number of detectable kisspeptin cells **(A)** or silver grains per cell **(B)**. Darkfield photomicrographs of representative POA sections from normal weight **(C)**, lean **(D)** and lean + leptin treated **(E)** OVX ewes at 10X magnification. Brightfield photomicrographs of representative kisspeptin cells in the POA of normal weight **(F)**, lean **(G)** and lean + leptin treated **(H)** OVX ewes at 40X magnification. Data are means ± SEM. *$p<0.05$, **$p<0.01$.

4.4.2 Experiment 2 – Expression of Ob-Rb in Kisspeptin neurons of the ARC and POA

The single ARC kisspeptin cell as well as the 50 ARC and 20 POA kisspeptin cells all expressed the leptin receptor Ob-Rb (Figure 4.3). When corrected for malate dehydrogenase, Ob-Rb mRNA levels were 1.47, 1.52 and 0.21 for 50 ARC, 20 POA and the single ARC kisspeptin cell respectively.

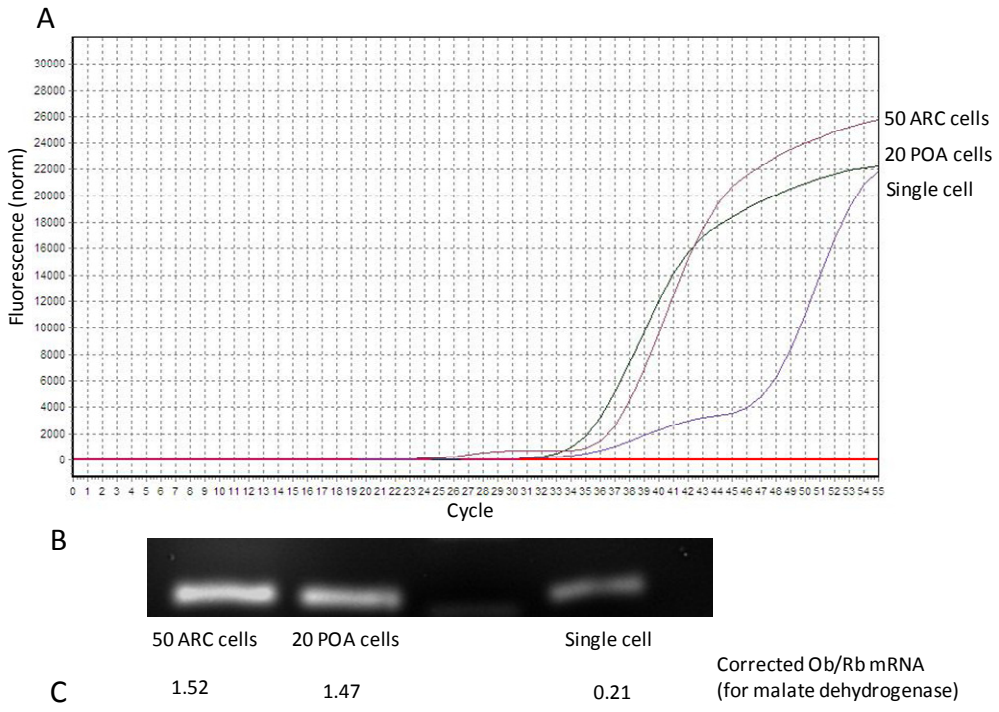

Figure 4.3: Kisspeptin cells of the ARC and the POA express the long form leptin receptor Ob-Rb. **(A)** RT-PCR amplification of 50 ARC, 20 POA, and a single kisspeptin acquired by laser capture microdissection. **(B)** Ob-Rb expression on 50 ARC, 20 POA and single ARC cell from PCR DNA run on 2% agarose gel. **(C)** Corrected Ob-Rb values for malate dehydrogenase**.**

4.4.3 Experiment 3 - Reciprocal connections between kisspeptin and POMC/NPY cells

Kisspeptin fibres were seen in close apposition with 7.8±2.5% and 7.1±1.1% of NPY cells in intact and OVX ewes respectively (Figure 4.4). Close apposition of kisspeptin fibres was also with 22.9±4.6% and 18.1±1.8% of POMC cell bodies (intact and OVX ewes respectively). Total average (intact and OVX) contacts with kisspeptin-immunoreactive varicose fibres were 20% (POMC) and 7.4% (NPY). Kisspeptin fibres come into contact with POMC cell bodies more frequently than NPY cell bodies in intact ($p<0.05$) and OVX ewes ($p<0.01$; Figure 4.4 G).

NPY fibres were seen in close apposition with 13.9±1.3% and 29.1±3.1% of kisspeptin cells in intact and OVX ewes respectively (Figure 4.4). POMC fibres were seen in close apposition with 44.1±4.9% and 32.3±6.9% kisspeptin cell bodies (intact and OVX ewes respectively). POMC fibres came into contact with kisspeptin cells more frequently than NPY cell bodies in OVX ewes ($p<0.01$; Figure 4.4 H). NPY fibres made close appositions with kisspeptin cells more frequently in OVX ewes than in intact ewes ($p<0.01$; Figure 4.4 H).

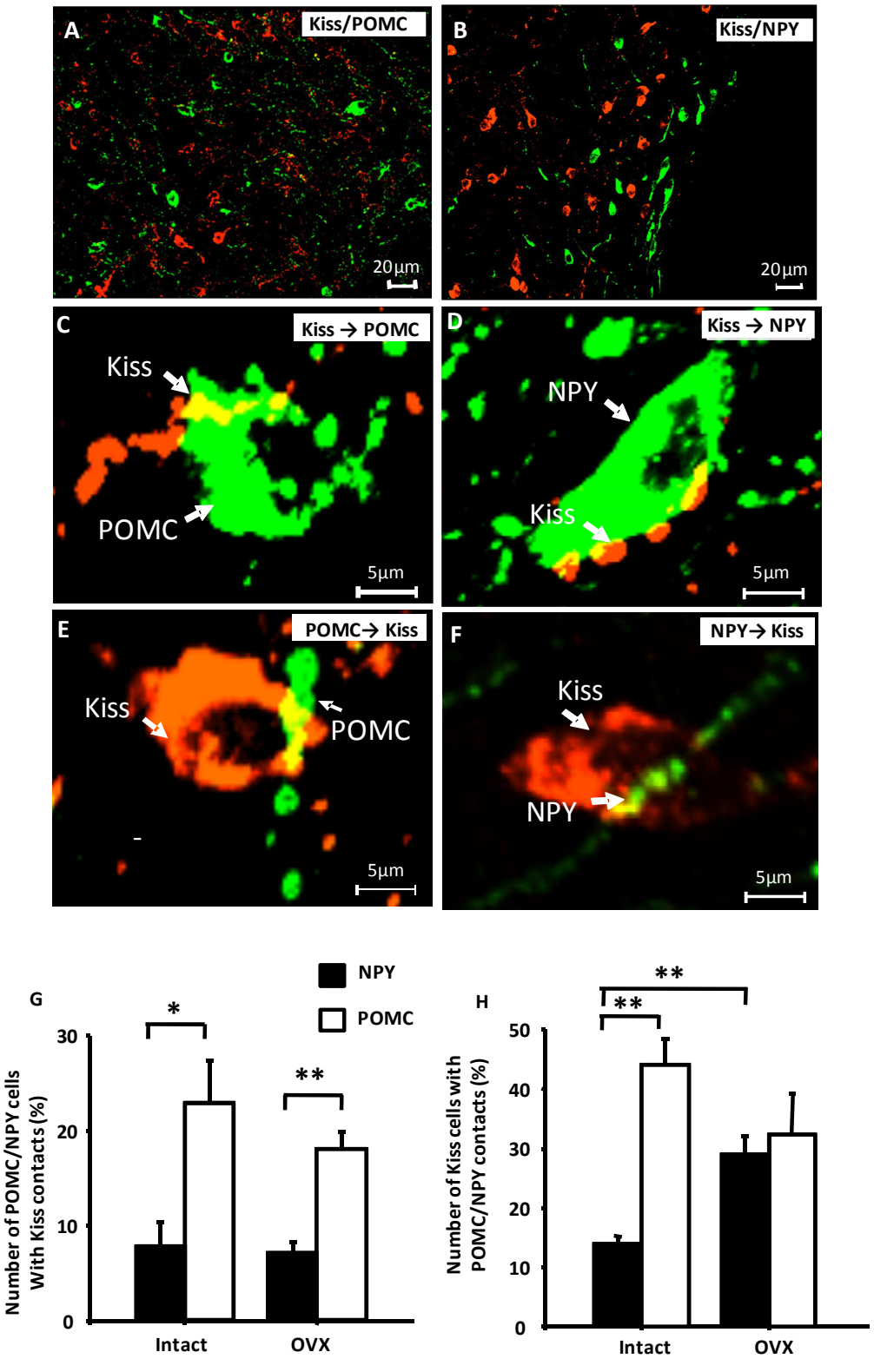
A
Kiss/POMC
20µm
B
Kiss/NPY
20µm
C
Kiss → POMC
Kiss
POMC
5µm
D
Kiss → NPY
NPY
Kiss
5µm
E
POMC→ Kiss
Kiss
POMC
5µm
F
NPY→ Kiss
Kiss
NPY
5µm
G
NPY
POMC
Number of POMC/NPY cells With Kiss contacts (%)
30
20
10
0
*
**
Intact
OVX
H
Number of Kiss cells with POMC/NPY contacts (%)
50
40
30
20
10
0
**
**
Intact
OVX

Figure 4.4: Kisspeptin fibres come into close contact with POMC and NPY cells, as do POMC and NPY fibres with kisspeptin cells. **(A)** Low power fluorescent photomicrograph indicating POMC (green) and Kisspeptin (red) staining in the arcuate nucleus. **(B)** NPY (green) and Kisspeptin (red) in the arcuate nucleus at low power. Digitally magnified image of a Kisspeptin fibre coming in close apposition with a POMC cell **(C)** and NPY cell **(D)**. Digitally magnified image of a POMC **(E)** and NPY **(F)** fibre coming in close contact with kisspeptin cell**s**. Average percentage of kisspeptin fibres contacting NPY and POMC cells in the ARC of intact and OVX ewes **(G)**. Average percentage of POMC and NPY fibres contacting kisspeptin cells in the ARC of intact and OVX ewes **(H)**. Data are means ± SEM. *p<0.05, **p<0.01.

4.4.4 Experiment 4 –Effect of Kisspeptin treatment on POMC and NPY gene expression

IIIV infusion of Kisspeptin to OVX ewes increased ($p<0.01$) NPY mRNA expression in the ARC in terms of the number of detectable cells and the level of expression/cell (Figure 4.5 A-F). Kisspeptin reduced POMC mRNA expression in the ARC by decreasing both the number of detectable cells ($p<0.05$) and the level of expression/cell ($p<0.01$ compared to vehicle treated controls; Figure 4.5 G-H).

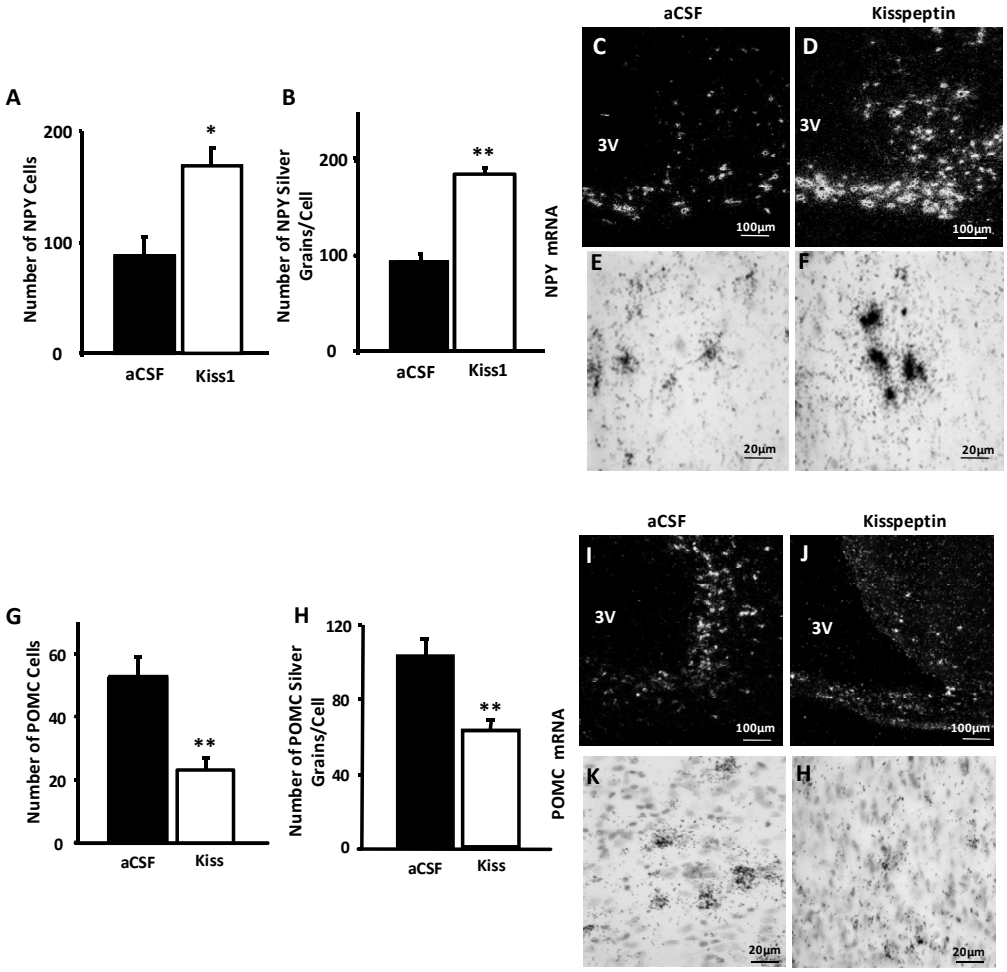

Figure 4.5: Effect of third ventricular infusion of aCSF or Kisspeptin (5µg/h) for 20h on NPY and POMC gene expression in the ARC of OVX ewes, expressed by the average number of cells per section and the number of silver grains per cell **(A-B, G-H)**. Darkfield (10X) photomicrograph representations of aCSF treated **(C,I)** and kisspeptin treated **(D,J)** and brightfield (40X) of aCSF treated **(E,K)** and Kisspeptin treated **(F,H)**. aCSF treatment represented by solid bar; Kisspeptin treatment represented by open bar. Data are means ±SEM. *p<0.05, **p<0.01.

4.5 Discussion

The results of this study provide strong evidence that kisspeptin cells are regulated by leptin. This is most likely to be a direct effect on these cells in both the ARC and POA, since both populations express Ob-Rb. Moreover, we present evidence of complex reciprocal relationships between kisspeptin cells and POMC/NPY cells in the ARC which may synchronise the regulation of systems within the brain that regulate metabolic function as well as reproduction. We substantiate the notion that kisspeptin regulates POMC and NPY cells by demonstration of direct effect on gene expression, allowing a means by which kisspeptin cells that are highly responsive to estradiol-17β might modulate 'appetite-regulating' systems (NPY and POMC cells).

Our data shows a reduction in Kiss1 gene expression in lean sheep, which is partially restored by leptin. These findings confirm and extend previously generated data in both rats and mice (Castellano *et al.* 2005; Smith *et al.* 2006a). Thus, in our model of the lean hypogonadotropic OVX ewe, which is a state in terms of metabolic condition (Henry *et al.* 2000) but has reduced energy stores in terms of adipose tissue, Kiss1 mRNA expression is reduced in cells of both the ARC and the POA. Previous studies concentrated on the kisspeptin cells of the ARC in the mouse (Smith *et al.* 2006a), but our evidence of effect on the kisspeptin cells of the POA are important in terms of control of GnRH cells by kisspeptin. This is because there is no evidence of direct input to GnRH cells from cells of the ARC (Pompolo *et al.* 2001). Thus, it is most likely that the high level of direct kisspeptin input to GnRH cells (Smith *et al.* 2008a) originates from the kisspeptin cells located in the POA, so the present data showing regulation of this subset of cells by altered body weight and leptin assumes significance. Regulation of GnRH cells by kisspeptin cells of the ARC may be effected by inter-neuronal pathways, since cells in various regions of the POA and BnST project directly to the GnRH cells (Pompolo *et al.* 2005).

The lowered level of Kiss1 expression in lean animals may be at least one cause of the hypogonadotropic state, since kisspeptin has a potent stimulatory effect on GnRH secretion (Thompson *et al.* 2004; Caraty *et al.* 2007). This is supported by

the demonstration that leptin treatment is able to partially restore the expression of Kiss1, as well as pulsatile secretion of LH in the hypogonadotropic lean animals (Backholer *et al.* In Press-a). On the other hand, we have previously shown that POMC gene expression and melanocortin peptide levels are lowered in our lean ewe model, and leptin treatment restores the melanocortin system to normal, in concert with restoration of gonadotropic function (Backholer *et al.* In Press-b). In this earlier paper we present strong evidence that the melanocortin system is the means by which leptin communicates with GnRH cells, as GnRH cells do not express Ob-Rb (Finn *et al.* 1998; Hakansson *et al.* 1998; Quennell *et al.* 2009). Given that an equivalent leptin dose fully restores POMC gene expression, in contrast to partially restored Kiss1 gene expression, it seems possible that the transmission of leptin signalling to GnRH cells is predominantly through the melanocortin system. Apart from kisspeptin originating from the POA acting directly on the GnRH cells, and the kisspeptin cells of the ARC acting indirectly, the ARC kisspeptin cells may also act to facilitate melanocortin transmission of metabolic information to GnRH cells (see figure 4.6 for summary model of the integrated function of kisspeptin neurons to regulate reproduction and metabolic function).

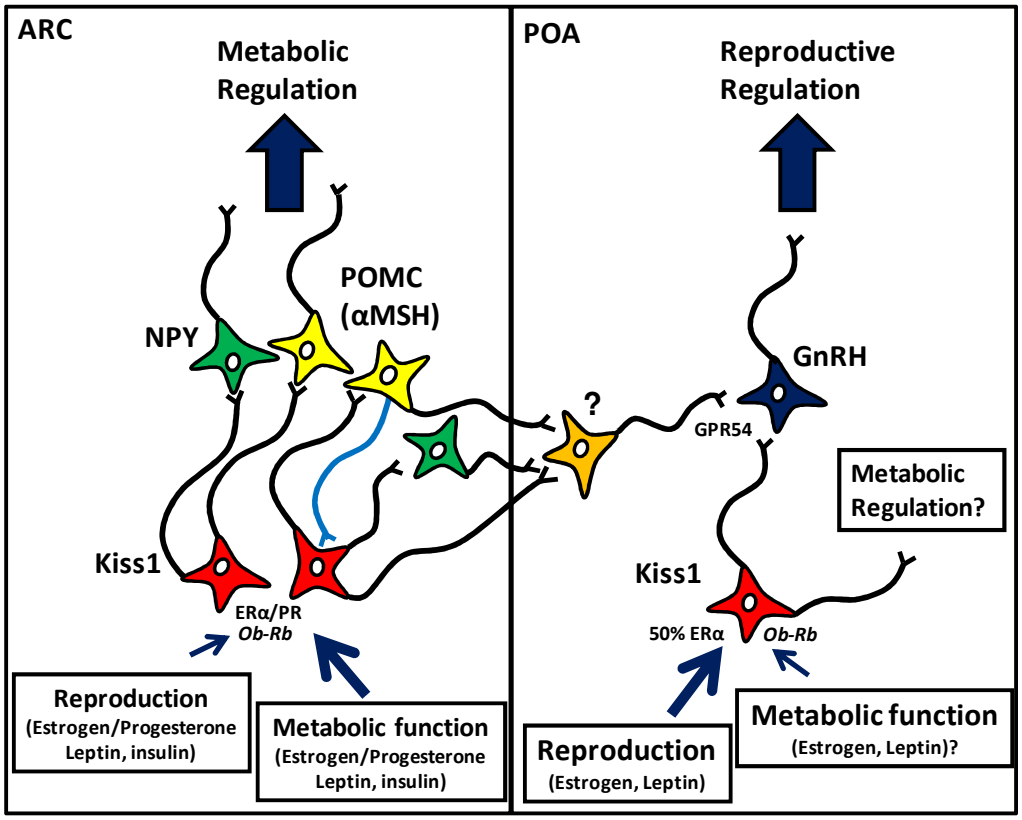

Figure 4.6: Model of the integrated function of kisspeptin neurons to regulate reproduction and metabolic function. **ARC:** In the ARC, kisspeptin cells possess estrogen receptors (ERα), progesterone receptors (PR), and leptin receptors (Ob-Rb). These cells may also possess other receptors that detect metabolic signals (insulin, ghrelin, etc). By sensing leptin and sex steroid levels, the kisspeptin cells may relay information to NPY and POMC cells (black lines). NPY and POMC cells possess leptin receptors (and a subset possess ERα) and reciprocal connections (blue) provide possible regulation of the reproductive system through kisspeptin cells. It is also known that melanocortins stimulate the reproductive axis (Backholer *et al.* In Press-a) and kisspeptin cells provide synaptic input to kisspeptin cells in the ARC, so both melanocortin cells and kisspeptin cells may act in concert to control GnRH cells. Regulation of GnRH cells by kisspeptin and melanocortin cells of the ARC occurs via at least one inter-neuron (Pompolo *et al.* 2001; Backholer *et al.* In Press-b). In addition to controlling reproduction, the integrated kisspeptin/NPY/melanocortin system of the ARC may regulate food intake and energy expenditure. Thus, effects of leptin may be directly upon NPY/POMC cells or indirectly via kisspeptin cells. Furthermore, the sex steroid effects to regulate food intake and energy expenditure may be relayed via kisspeptin cells, which express the relevant receptors at high level. **POA:** Kisspeptin cells express Ob-Rb and 50% of these cells posses ER-α. The kisspeptin cells in the POA may provide direct input to GnRH cells (which posses GPR54) to regulate reproduction. By action on the kisspeptin cells in this region of the brain, estadiol-17β and leptin may control reproductive function. It is also known that cells in this region of the brain control homeostatic processes (Dagnault and Richard 1997), so it cannot be ruled out that kisspeptin cells are important in this regard.

A possible reason as to why we only achieved partial restoration of Kiss1 gene expression in lean animals given leptin may be that other metabolic factors in addition to leptin also provide feedback information of metabolic status. These include insulin and glucose (Miller *et al.* 1998; Parton *et al.* 2007; Szymanski *et al.* 2007), ghrelin (Wren *et al.* 2001; Kurose *et al.* 2005) and fatty acids (Tapsell *et al.* 2009), which are known to act on the cells of the hypothalamus. Our lean OVX ewe model is characterised not only by a reduction in leptin levels but also lowered insulin concentrations (Henry *et al.* 1999; Kurose *et al.* 2005), although glucose and non-esterified fatty acid levels in plasma are similar to those in normal animals (Henry *et al.* 2000). In this model leptin treatment does not change insulin levels (Henry *et al.* 1999) so this is unlikely to be a significant factor. Other recent studies show that leptin, but not insulin, stimulates Kiss1 mRNA expression in mouse hypothalamic cell lines (Luque *et al.* 2007) and male diabetic rats (Castellano *et al.* 2006). Earlier studies from this laboratory showed a trend towards an increase of leptin receptor (Ob-Rb*)* gene expression in the ARC of the lean OVX ewe when compared to normal weight controls (Kurose *et al.* 2005), so adequacy at this level is not likely to be a factor limiting leptin signalling. Ghrelin levels are reduced in the lean OVX ewe (Kurose *et al.* 2005) but it is unknown whether this hormone affects Kiss1 levels.

Corroborating data of the mouse (Smith *et al.* 2006a), Ob-Rb was found on ARC kisspeptin cells of the ewe. Furthermore, at least some of the cells previously observed to express Ob-Rb in the POA of the ovine brain (Williams *et al.* 1999) appear to be those that produce kisspeptin. There is little doubt that kisspeptin cells in both the ARC and POA of the ewe brain positively regulate GnRH cells, based on direct evidence of the central action of kisspeptin to stimulate GnRH and LH secretion (Irwig *et al.* 2004; Thompson *et al.* 2004; Caraty *et al.* 2007) and up regulation of both subsets of cells in the late follicular phase of the estrous cycle, prior to the preovulatory surge in GnRH/LH secretion (Estrada *et al.* 2006). In addition, an estradiol-17β stimulus that produces a surge in GnRH/LH secretion in the OVX ewe produces a robust fos response in cells of the ARC (Smith *et al.* 2006c), consistent with earlier work showing that the region of the brain in which estrogen acts to cause the surge is within the ARC/ventromedial hypothalamus

(Blache *et al.* 1991; Caraty *et al.* 1998). This is likely to be through at least one interneuronal chain as we have found that very few ARC kisspeptin cells project to the ventromedial POA (Backholer *et al.* In Press-b). It is possible that the ARC kisspeptin cells do however project to the dorsolateral POA, from which there are direct connections to the GnRH cells (Pompolo *et al.* 2005) and this is where kisspeptin neurons are found. The demonstrated presence of Ob-Rb expression in kisspeptin neurons of the POA provides a novel conduit for leptin regulation of GnRH/gonadotropin secretion.

We have presented evidence that POMC and NPY cells project to kisspeptin cells in the ARC, which is important because these pathways may provide an indirect conduit to GnRH cells. With respect to melanocortin production by POMC cells and the stimulatory effect on the reproductive axis (Backholer *et al.* In Press-b), an effect via kisspeptin cells is one possible conduit, in addition to the possible role of orexin cells (Backholer *et al.* In Press-b). Notably, over 40% of kisspeptin cells receive input from melanocortin cells. This allows the melanocortin cells, 100% of which express Ob-Rb, to influence kisspeptin cells, which could further explain the leptin effect on the latter and may serve to amplify the effect of leptin on kisspeptin cells, which is then transmitted to GnRH cells. The observation that melanocortin input to kisspeptin is greater than that from NPY cells, suggests that the melanocortin cells are fundamentally more important in synchronising effects of peripheral signalling agents on the metabolic and reproductive systems of the hypothalamus.

In addition, melanocortin cells were seen to receive a higher level of input from kisspeptin cells than NPY cells, again emphasising the importance of the former cell type. The observation that kisspeptin treatment reduced POMC expression and increased NPY expression substantiates the histochemical finding. Since almost 100% of ARC kisspeptin cells express estrogen receptor-α (ER-α) (Franceschini *et al.* 2006) and progesterone receptor (PR) (Smith *et al.* 2007), but only a small subset of POMC (Lehman and Karsch 1993) and NPY (Skinner and Herbison 1997) cells express sex steroid receptors, the ARC kisspeptin neurons may allow significant sex steroid regulation of NPY and POMC cells, which may be a mechanism to regulate

metabolic systems as well as reproduction. If this nexus were part of an intricate feedback system to regulate the reproductive system, one might expect some degree of synaptic plasticity regarding the extent to which kisspeptin fibres contact POMC and NPY cells, but we did not find any differences between gonad-intact and ovariectomised ewes. The novel finding that kisspeptin regulates POMC and NPY cells provides a means by which the well known effects of sex steroids on food intake and energy expenditure (Parker *et al.* 2001; Asarian and Geary 2002; Shi and Clegg 2009) might be caused. In human females, food intake peaks in the luteal phase of the estrous cycle when estrogen is at its lowest and is at nadir during the peri-ovulatory period when estrogen level reach peak concentrations (Buffenstein *et al.* 1995). Recent research however implies that the kisspeptin system is not involved in the regulation of food intake. Acute kisspeptin treatment of lean animals at a dose effective to increase LH secretion does not alter food intake (7, 34) and GPR54 knockout mice display no obvious phenotype of metabolic disruption (de Roux *et al.* 2003). These studies however investigate the effect of short term kisspeptin treatment on food intake and a more sustained infusion of kisspeptin may be needed. Such studies are in progress in our laboratory at present.

In summary, we demonstrate that leptin status regulates Kiss1 mRNA in the ARC and POA, and these cells express Ob-Rb. Furthermore, we present a novel reciprocal interaction between the ARC kisspeptin cells and the POMC and NPY cells. This circuitry may provide a conduit through which sex steroids and leptin can act to regulate reproductive and metabolic homeostasis.

4.6 Acknowledgements

We thank Mr B. Doughton, Ms L. Morrish, and Ms E. Gleeson for their technical assistance.

Chapter 5
General Discussion

5.1 Major Findings

The work presented in this thesis implicates the melanocortin system as an eminent positive regulator of the reproductive neuroendocrine system. Specifically, Chapter 2 demonstrates that leptin and melanocortin treatment is able to 'trick' the brain into 'thinking' body energy stores are sufficient to ameliorate pulsatile LH secretion in hypogonadotropic lean ewes. Leptin treatment causes an up regulation of POMC mRNA expression which may result in an increase in the levels of acetylated α-MSH in the terminal beds, as the levels of this acetylated peptide is altered under differing leptin status. Chapter 3 further demonstrates the stimulatory role of the melanocortin system on GnRH neurons in times of reproductive suppression, but suggests that this occurs indirectly via a proposed novel interneuronal pathway. During the luteal phase at least, this is likely to involve the activation of ORX cells in the DMH and the kisspeptin cells in the POA. Chapter 4 confirms and extends data from rodent species showing that the kisspeptin system is metabolically regulated under altered leptin status. Preliminary data demonstrate the presence of the long form leptin receptor, Ob-Rb, on populations of Kisspeptin neurons in the ARC and POA and therefore a direct effect of leptin on these cells is likely. Interestingly, this study also demonstrates a novel reciprocal synaptic connection between the kisspeptin and POMC/NPY systems in the ewe, and central treatment with kisspeptin down regulates POMC mRNA and up regulates NPY mRNA. The mutual communication between these systems may aid in the transmission of sex steroids and leptin to regulate reproductive and metabolic function within the brain (see Figure 4.6 for proposed central transmission of sex steroids and leptin via kisspeptin/POMC/NPY systems). The higher level of synaptic input between the kisspeptin and POMC

systems illustrate the importance of the POMC cells, and therefore melanocortins, in signalling peripheral signals on metabolic status to the brain.

5.2 Future Directions and limitations

The body of work presented in this thesis is not without limitations. In chapter 2 we speculate that melanocortins act down stream to leptin signalling which is predicated on the findings that leptin up regulates POMC mRNA and that melanocortin receptor activation is capable of stimulating LH release. In order to conclusively attest this supposition that a series relationship exists between leptin and the melanocortins, further studies should look at treating lean hypogonadotropic ewes with leptin in the presence of a MC3-R/MC4-R receptor antagonist. Rat studies have indeed shown that the melanocortin antagonist SHU9119 decreases the magnitude of the leptin induced LH surge in the 3-day starved rat (Watanobe *et al.* 1999a).

The most salient observation in chapter 2 was that low leptin status resulted in a decrease of acetylated α-MSH in the terminal beds of the POA, and in areas of the hypothalamus outside of the ARC. These results illustrate a line of reasoning that it is not sufficient to simply measure POMC gene expression and/or α-MSH peptide concentration in the ARC, and a full post-translational profile of the melanocortins in the brain is required. It would be instructive to further examine acetylated α-MSH levels in the terminal beds of lean animals treated with leptin to ensure direct leptin regulation of this post-translational peptide. Our sheep model takes six months to generate and extensive experimental work is required for this type of analysis. For this reason we first wished to know whether acetylation was an important issue, which we now believe to be the case. It seems appropriate to interrogate this system further and future work should be directed towards profiling post-translational forms of α-MSH in lean hypogonadotropic ewes, treated with and without leptin. It would be further informative to identify the enzyme(s) responsible for the acetylation process as therapeutic intervention may target the acetylation of melanocortins.

Chapter 3 identified a novel conduit by which melanocortins may regulate GnRH cells. A clear physiological role for the ORX neurons in the DMH of the ewe is yet

to be determined, and these cells should perhaps be subject to a more thorough investigation in the control of reproduction and energy balance in this species. Likewise, the kisspeptin cells of the POA remain relatively enigmatic, but may provide the missing link between the melanocortins and GnRH neurons in relation to both leptin and sex steroid feedback. To further delineate this neuronal circuitry, *in vivo* studies should investigate the effect of a melanocortin agonist on LH secretion in the presence of a kisspeptin or ORX antagonist. *In vitro* studies should examine the presence of melanocortin receptors on ORX and kisspeptin cells in the dorso-lateral POA. Additionally, more extensive anterograde and retrograde tracer studies should be undertaken to confirm the projection of kisspeptin neurons in the POA to GnRH neurons. Chapter 3 additionally demonstrates that melanocortin treatment is able to overcome the negative feedback effect of progesterone in the luteal phase of the estrous cycle to increase basal LH levels. The levels of acetylated α-MSH across the ovine estrous cycle is unknown, and the increase in LH levels in luteal phase ewes by the melanocortin agonist, may have transpired as a result of increased levels of acetylated α-MSH. Future work in this area should profile the post-translational changes of the melanocortins across the estrous cycle and in the non-breeding season to determine if sex-steroids regulate acetyltransferase activity. Finally in this chapter, melanocortin treatment to seasonal anestrous ewes was unable to induce an LH surge or cause ovulation, but was able to overcome the enhanced effect of estrogen negative feedback that prevails during anestrous (Legan *et al.* 1977) to increase basal LH secretion. It is possible that the dose used in our study was only sufficient to prime the ovarian follicles, and a subsequent dose is needed to see an effect on ovulation. Further investigation is warranted to determine alternative doses and modes of melanocortin agonist delivery.

The data presented in chapter 4 demonstrates putative synaptic input of kisspeptin fibres to NPY and POMC cells using immunohistochemistry and Zeiss Z-stack apotome analysis. In addition, *in situ* hybridisation demonstrated regulation of NPY and POMC cells after central kisspeptin treatment. This novel regulation of ARC NPY and POMC gene expression by kisspeptin treatment in the ewe, opens the door to a great deal of further research expounding the role of the kisspeptin

system in regulating metabolic homeostasis. In this respect, it would be interesting to identify the role, if any, that the kisspeptin system has in regulating thermogenesis and/or food intake.

Chapter 4 also illustrates a down regulation of Kiss1 mRNA expression in lean hypogonadotropic ewes, with only partial restoration by leptin treatment. This suggests that multiple factors regulate the kisspeptin system in times of metabolic insufficiency, such as insulin, glucose and ghrelin. It would be useful to delineate which other metabolic factors regulate this system and the mechanisms behind this regulation. Furthermore, RF-amide related peptide 3 (RFRP-3), a recently discovered RF-amide, appears to be important in the control of the reproductive neuroendocrine axis, acting in concert (with opposing effects) with kisspeptin (Smith *et al.* 2008a). There are demonstrable effects of this peptide on food intake in rats (Johnson and Fraley 2008) and the RFRP-3 system in the ovine brain projects to known appetite regulating cells (Qi *et al.* 2009b). Thus, it would be interesting to determine if the expression of this newly discovered peptide is also altered with body weight and leptin status.

The melanocortins are instrumental in the restoration of the reproductive axis in lean animals treated with leptin, and the kisspeptin system may act in concert with this. The data presented in this thesis presents a novel reciprocal interaction between the POMC and kisspeptin cells, and we propose that these latter cells facilitate the actions of the melanocortins to regulate metabolic and reproductive homeostasis. It is necessary to expand this preliminary work further in the ewe, as well as in other animal models, to substantiate our current results and gain a more comprehensive understanding into the way these systems communicate.

Although the studies carried out in this thesis focused on the melanocortin α-MSH, the melanocortin γ-MSH has also been found to stimulate the reproductive axis (Stanley et al. 2003), and central administration of β-MSH to rats has been found to be at least as effective as α-MSH in suppressing food intake (Kask et al. 2000). Unlike the sheep or humans, rodents do not posses the paired lysine cleavage sites to produce β-MSH (Pritchard et al. 2002), thus this melanocortin product remains somewhat forgotten in the current research. One study indeed suggests that β-

MSH has a greater affinity for the MC4-R than α-MSH, thus it seems valuable to investigate this melanocortin sub-type further. β-MSH is produced in the sheep, therefore this species would be an ideal model to interrogate this system further, and it may certainly prove to be, that β-MSH plays a more important role in this line of study.

5.3 Clinical Implications

This thesis implicates the use of a melanocortin agonist in the therapeutic intervention of hypothalamic amenorrhea, however further clinical implications may evolve with new research. For example, additional uses of melanocortin treatment may be extended to infertile males. Males also secrete LH which is necessary for testosterone production and thus spermatogenesis, and when LH and testosterone concentrations are compromised, so too is fertility. Previous studies have demonstrated that treatment with a melanocortin agonist effectively increases LH levels in male rats (Stanley et al. 2003), therefore it is possible that melanocortins may restore or enhance reproductive capabilities in men whose reproductive system is compromised by lowered testosterone production. Furthermore, females with endometriosis display decreased levels of gonadotrophins and estrogen concentrations (Trinder and Cahill 2002) and the melanocortin system may provide therapeutic implications for treatment of this condition.

The use of melanocortins in stimulating the reproductive axis may also be extended to livestock to increase breeding activity in times of reproductive suppression as seen in the anestrous season. Although the data presented in this thesis showed that the melanocortin agonist MTII was unable to induce ovulation in the anestrous season of ewes, it does not preclude a therapeutic possibility. As mentioned above, a higher dose may be required to translate the increase in basal LH levels to an LH surge, ovulation, and the formation of a corpus luteum.

In the condition of hypothalamic amenorrhea circulating levels of leptin are reduced and leptin treatment is able to enhance the reproductive axis in human females (Welt et al. 2004). Although current clinical research is indeed investigating the use of leptin as a therapeutic intervention into hypothalamic

amenorrhea, leptin is a large protein hormone and continuous administration with this hormone may lead to the production of antibodies. Furthermore, the manufacture of leptin by recombinant technology would be expensive when producing leptin in commercial quantities. The use of GnRH mini pumps have also been investigated as a therapeutic intervention, however these pumps are quite cumbersome to the individual and attendant problems may occur. Accordingly, the need still exists for an alternative method of restoring the hormonal balance of the hypothalamo-pituitary-gonadal axis.

A melanocortin therapeutic presents as a promising target in addressing hypothalamic amenorrhea, however further research is needed to substantiate its transition into clinical trial research. Melanocortins have been found to increase blood pressure and heart rate (Nordheim *et al.* 2006), and the need exists for the development of specific agonists which minimise adverse effects on the cardiovascular system.

5.4 Concluding remarks

Without a clear mechanistic explanation for the neuropeptide aberrations associated with hypothalamic reproductive suppression, and further clarification of the pathophysiology, an effective therapeutic target is doubtful. This thesis substantiates a growing body of work in smaller animal models, and adds further impetus to our understanding of the relationship between neuroendocrine reproductive function and metabolic status. This work not only promotes the utility of the melanocortins or the acetylation enzymes (once identified) as targets for therapeutic intervention for hypogonadotropic hypogonadism, but also lends support to the use of other novel therapeutic approaches involving the kisspeptin and/or ORX systems. It is anticipated that future efforts in this field will see a transition from bench to bed research and ultimately aid in the prevention of osteoporosis, stress fractures and infertility as a result of lowered circulating sex steroids associated hypothalamic amenorrhea.

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