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ROLE OF DOPAMINE D2 RECEPTORS IN THE NEURAL CIRCUITRY UNDERLYING SEASONAL REPRODUCTION IN THE SHEEP

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Abstract

Seasonal reproduction in sheep is determined by changes in the responsiveness of GnRH neurons to the negative feedback action of estradiol, mediated by dopaminergic afferents and dopamine D2 receptors (D2R). It is not known whether this influence acts directly onto GnRH neurons or indirectly via interneurons such as KNDy cells. In anestrous ewes, immunocytochemistry revealed D2R expression in ~50% of GnRH neurons independent of region. In contrast, using dynorphin as a marker, D2R colocalized >80% of KNDy cells and <10% of dynorphin cells of the POA. No seasonal variation was found in D2R expression in GnRH cells; however colocalization significantly increased in KNDY cells in anestrous *vs.* breeding season. Immunochemistry found that kisspeptin fibres contact GnRH neurons synaptically. Results suggest that seasonal changes in this circuit are mediated by dopaminergic afferents contacting D2R-containing KNDy neurons, which in turn decreases expression of the stimulatory neuropeptide kisspeptin, inhibiting GnRH in seasonal anestrous.

Keywords: GnRH, Dopamine D2 Receptor, kisspeptin, KNDy, estradiol negative feedback, seasonality, dynorphin, hypothalamus, reproductive neuroendocrine system, sheep, immunocytochemistry, confocal microscopy.

Co-Authorship Statement

M. J. Maltby performed all experimental work and statistical analysis in this thesis except for the following:

Erinna Brown, an undergraduate Honours student of the BMSc program, performed immocytochemistry on one series of tissue, as part of her undergraduate thesis. Analysis of these tissues was performed by me and is included in experiment 1.

Katherine Bateman, an undergraduate Honours student of the BMSc program, performed immocytochemistry on one series of tissue, in partial completion of her undergraduate thesis. Analysis of these tissues was performed by me and is included in experiment 3.

Xu Wang, Research Assistant in the laboratory, performed protein isolation procedures and assisted with Western Immunoblotting protocols.

Dr. Lique Coolen performed the microdissection of arcuate nucleus of fresh frozen tissue that was used for the Western blot.

Dr. Robert Goodman, collaborator at the University of West Virginia, and his staff were responsible for all procedures using the animals, including the local microinjection of quinpirole in experiment 2, and all tissue collection for all experiments.

Dr. Michael Lehman designed all experiments and provided supervision.

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List of Abbreviations

AHA	Anterior Hypothalamus
APS	Ammonium Per Sulphate
ANOVA	Analysis of Variance
cAMP	Cyclic Adenosine Monophosphate
D1R	Dopamine D1 Receptor
D2R	Dopamine D2 Receptor
DA	Dopamine
ddH_2O	Double Distilled Water
E_2	Estradiol
ERα	Estrogen Receptor Alpha
FSH	Follicle Stimulating Hormone
GABA	γ-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GnIH	Gonadotropin-inhibiting Hormone
GnRH	Gonadotropin-releasing Hormone
GRK	G Protein-Coupled Receptor Kinase
GPR54	G Protein coupled Receptor 54
GSK-3	Glycogen synthase kinase 3
LH	Luteinizing Hormone
MBH	Mediobasal Hypothalamus
mRNA	Messenger Ribonucleic Acid
NKB	Neurokinin B
NPY	Neuropeptide Y
OC	Optic Chiasm
OFQ	Orphanin FQ
PAGE	Polyacrylamide Gel Electrophoresis
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PMR	Premamillary Region
POA	Preoptic Area
PSA-NCAM	Polysialylated Form of Neural Cell Adhesion Molecule
PVDF	Polyvinylidene Fluoride
RCh	Retrochiasmatic Area
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of Mean
SON	Supraoptic Nucleus
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TEMED	Tetramethylethylenediamine
TH	Tyrosine Hydroxylase
Vglut-1	Vesicular Glutamate Transporter 1
vmPOA	Ventromedial Preoptic Area

Chapter 1

Introduction and Background

1.1 Introduction

Seasonal reproduction is a strategy used by many animals to ensure that offspring are born during environmentally favourable conditions. In order to control the timing of reproduction, females of many mammalian species undergo periods of suppressed ovarian function primarily by inhibition of the secretion of the highly conserved decapeptide, gonadotropin-releasing hormone (GnRH) (for review see Lehman *et al.* 1997, for a thorough overview of GnRH see Gore 2002). One such example is the sheep (*Ovis aries*), a model organism for studies of the reproductive neuroendocrine system, in which the timing of the breeding and non-breeding seasons is coordinated each year to ensure that young are born during the favourable conditions of spring. The reproductive system is also reversibly suppressed in women, both under normal conditions, such as occurring during lactation, and pathologically. This research aims to contribute to the understanding of the circuitry underlying reversible suppression of reproductive function using the ewe as a model system. Specifically, this research aims to delineate the neural pathways and receptors by which estradiol (E_2) modulates the secretion of GnRH during the non-breeding season (anestrous) in the ewe.

1.2 Reproductive suppression in women

Women undergo ovarian suppression under normal physiological conditions and pathologically. Normal ovarian suppression occurs from a reduced or absent secretion of gonadotropins between roughly age two and menarche when pulsatile GnRH secretion initiates puberty (for review see DiVall and Radovick 2008). Ovarian suppression also occurs during

pregnancy and lactation when it is undesirable to divide the limited energy provided by the mother to her current child. Ovarian suppression can also occur pathologically with numerous disorders contributing to abnormal secretion of gonadotropins. Malnutrition, seen in women with *anorexia nervosa*, may create dysfunction in the hypothalamic-pituitary system by reducing GnRH secretion manifesting itself in amenorrhea, and consequently, infertility (for review see Katz and Vollenhoven 2000). Failure of steroid feedback inhibition of GnRH secretion is thought to be responsible for elevated LH secretion in women with polycystic ovary syndrome, the leading cause of infertility among women, and leading hormonal disorder affecting approximately 6-8% of women of reproductive age (for review see Blank *et al.* 2006). Many other disorders concerning the control of the secretion of GnRH have been identified including central precocious puberty (for review see Partsch *et al.* 2002) and Kallmann syndrome (for review see Seminara *et al.* 1998). It is consequently of great importance to understand the fundamental nature of the neuroendocrine system involved in the secretion of GnRH in order to provide a rational basis for better treatments for reproductive disorders.

1.3 The sheep as a model system for the reproductive neuroendocrine system

The sheep (*Ovis aries*) is widely used as a model for neuroendocrine research. As a large mammal, the sheep offers many advantages over more traditional model organisms such as the rat. Due to the size of the sheep, it is possible to sample large quantities of blood without stress over prolonged periods providing for a more frequent and accurate measurements of endocrine events than is possible in smaller organisms. The sheep is a particularly useful model to study the reproductive neuroendocrine system as sampling of hormones can be combined with neuroanatomical manipulations of a large brain. In addition, sampling of GnRH is problematic in other species as peripheral blood cannot be used due to the short half-life of the hormone, but

can be measured via cannulation of the hypophyseal portal system in sheep (Clarke 2002). From an evolutionary perspective, the importance of GnRH secretion as the final common pathway in the higher order control of reproduction suggests that this aspect of the system remains highly conserved among mammalian species and consequently relevant for modelling human disease (Gore 2002). The reversible nature of the reproductive suppression in ewes also provides a model to study structural and functional neuroplasticity in the adult brain. Thus, the anestrous ewe is a useful model to study many aspects of reversible reproductive suppression.

1.4 Hypothalamic-pituitary-gonadal axis

GnRH neurons are the final common pathway in the brain responsible for modulating reproductive endocrine events. GnRH neurons in mammals are not clustered in a single nucleus of the hypothalamus, but rather are scattered in a distribution that, in the sheep, extends from the preoptic area (POA) to the mediobasal hypothalamus (MBH) (Lehman *et al.* 1986). GnRH is secreted into the hypophyseal portal system where it enters the anterior pituitary gland stimulating the release of two gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are conveyed by blood to the ovaries where they stimulate the biosynthesis and secretion of estradiol (E_2) and progesterone (Gore 2002). Once released, these sex hormones modulate a number of important physiological effects including regulation of the menstrual or estrous cycle. In the ewe, E_2 feeds back into the hypothalamus where it is involved in both inhibition of GnRH secretion in anestrous as well as the stimulation of GnRH secretion leading to the triggering of ovulation in the breeding season. The tight feedback control of the secretion of GnRH is therefore extremely important to reproduction in general. GnRH is secreted in two distinct modalities: surge and tonic secretion.

1.4.1 GnRH Surge

Although GnRH secretion is normally under the negative feedback action of E_2, the late follicular phase sees a profound increase in E_2 which causes a large sustained release of GnRH, the so-called "GnRH surge", into the hypophyseal portal system. The GnRH surge, in turn, induces an LH surge which is directly responsible for triggering ovulation. Studies examining transcriptional activation of GnRH cells using the immediate early gene marker Fos (Moenter *et al.* 1993), suggest that approximately half of all GnRH cells throughout the preoptic area and hypothalamus are responsible for the GnRH surge. Unlike the tonic secretion of GnRH (see below), the ability of E_2 to induce a GnRH surge does not vary with season (Lehman *et al.* 1997). Heightened E_2 is thought to induce a reduction in the ratio of inhibitory to excitatory neurotransmission onto GnRH neurons which in turn allows for large quantities of GnRH to be secreted and bind to simultaneously sensitized gonadotrophs (for review see Naftolin *et al.* 2007).

1.4.2 Tonic secretion of GnRH

GnRH is secreted into the hypophyseal portal system in a pulsatile manner throughout the breeding season with the pulse amplitude controlled by E_2 and the pulse frequency predominantly under the negative control of progesterone (for review see Goodman 1996, Goodman *et al.* 2002). The pulsatile nature of GnRH secretion is necessary to maintain release of the gonadotropins, as continuous infusion fails to restore normal LH secretion in monkeys with GnRH fibres destroyed by radiofrequency lesions in the arcuate nucleus and median eminence (Belchetz *et al.* 1978). A subset of GnRH cells located in the mediobasal hypothalamus is active during pulsatile secretion as determined by the expression of Fos (Boukhliq *et al.* 1999). GnRH cells become dramatically more sensitive to the negative feedback influence of low levels of E_2

during anestrous, inhibiting the frequency of LH pulses (Figure 1), and levels of E_2, so that ovulation can no longer be triggered (Lehman *et al.* 2002). This effectively shuts down reproduction during the anestrous; how this occurs at the level of the brain and GnRH system is still largely unknown and remains an area of active research.

1.4.3 Seasonality and the Role of Day Length

Seasonal variation in the ability of E_2 to inhibit GnRH secretion in ewes is controlled by daylength (photoperiod). Photoperiod is critical for reproductive function as simulating the short photoperiod of winter causes ewes normally in anestrous to ovulate by modulating response of GnRH to the E_2 negative feedback (Legan and Karsch 1980). Information about the ambient photoperiod is transduced by the retina to the pineal gland, through a multi-synaptic pathway that includes the suprachiasmatic nucleus and sympathetic preganglionic neurons in the spinal cord, to control a diurnal rhythm in the synthesis and secretion of the hormone melatonin. Melatonin, which is released during the nighttime, serves as a signal conveying daylength to the reproductive neuroendocrine system in sheep (Bittman *et al.* 1983); specifically, the duration of the nighttime rise in melatonin is responsible for regulating seasonal changes in the ability of E_2 to inhibit GnRH secretion. Studies involving microimplantation into selected brain sites have indicated that melatonin acts in the premamillary hypothalamic area to synchronize an annual rhythm of reproduction in the ewe (for review see Malpaux *et al.* 2002). However, the precise neural circuitry involved in relaying the melatonin signal to pathways mediating seasonal changes in E_2 negative feedback remains to be elucidated.

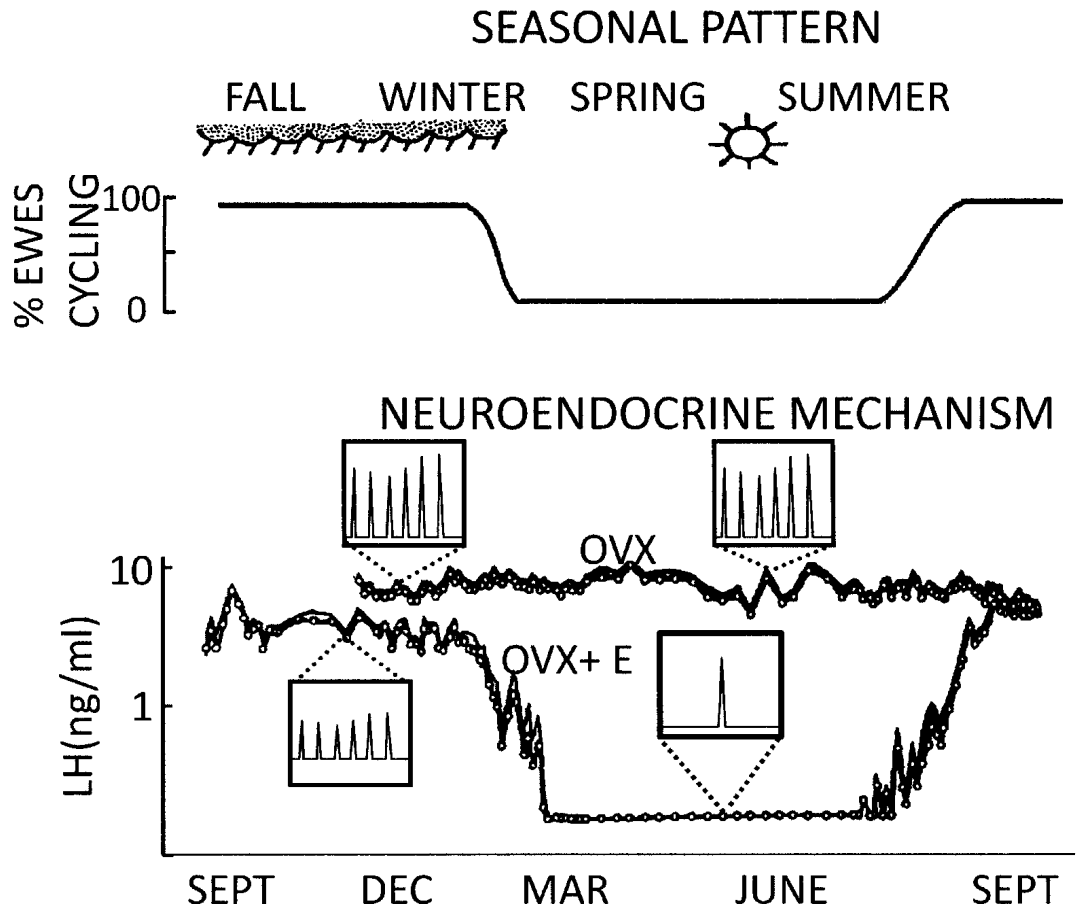


Figure 1 Neuroendocrine mechanism for seasonal anestrous in the ewe. Increased sensitivity of GnRH neurons to the negative feedback action of E_2 is responsible for decreased GnRH pulse frequency and consequently reproductive quiescence.

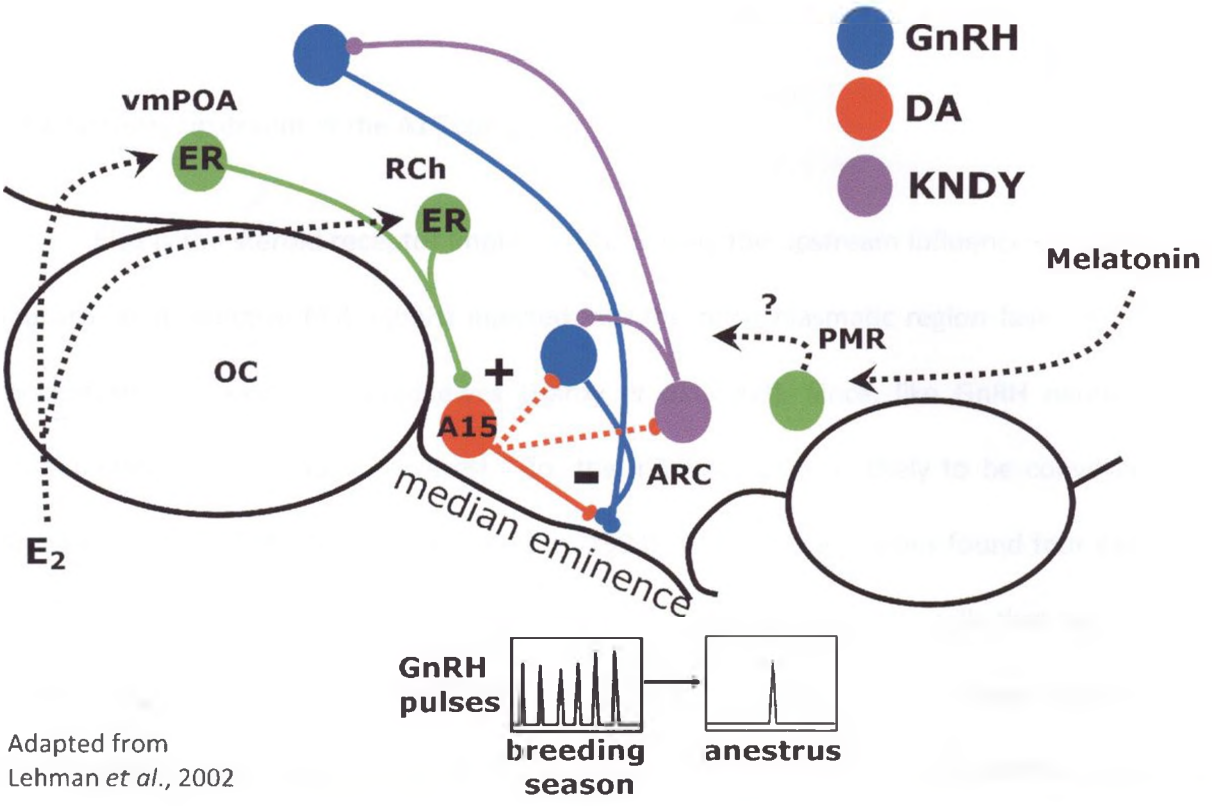
1.5 The estradiol negative feedback pathway

Seasonal reproductive transitions in sheep are a direct consequence of changes in the responsiveness of GnRH neurons to the negative feedback action of E_2. A marked increase in the sensitivity of GnRH neurons to the inhibitory effect of low levels of E_2 is responsible for decreased GnRH pulse frequency and consequently reproductive quiescence. As GnRH neurons do not express estrogen receptor alpha (ERα), the subtype of estrogen receptor required for negative feedback (Hardy *et al.* 2003), these changes are mediated by afferent inputs to GnRH cells rather than directly upon GnRH neurons themselves (Lehman and Karsch 1993, Lehman *et al.* 2002). The current working model for this circuitry is shown in Figure 2.

1.5.1 The A15 region

Substantial evidence implicates a group of dopaminergic neurons in the A15 region of the hypothalamus as being critical for conveying the inhibitory influence of E_2 upon GnRH neurons during the anestrous season. They have been shown to exhibit three characteristics necessary for this to be true: they inhibit GnRH secretion, they are functional in anestrous, and they are stimulated by E_2 (Goodman *et al.* 1996).

Dopamine was first implicated in the E_2 negative feedback pathway with the discovery that injecting the dopamine antagonist pimozide increased LH pulse frequency in anestrous ewes, at a time when LH is presumably inhibited by the negative feedback action of E_2, but did not alter LH pulse frequency in ovariectomized anestrous ewes or ewes in breeding season (Meyer and Goodman 1985). In addition, pharmacological studies using microinjections of pimozide into the retrochiasmatic area (RCh), but not the preoptic area (POA), stimulated GnRH secretion in anestrous ewes (Havern *et al.* 1991). Lesions of the A15 region caused an increase in LH pulse frequency only in E_2 treated anestrous ewes (Havern *et al.* 1994). Furthermore, A15 TH-



Adapted from
Lehman *et al.*, 2002

Figure 2 Hypothesized pathway for the E_2 negative feedback system during anestrous. ARC: arcuate nucleus; DA: dopamine; KNDy: Kisspeptin/neurokinin B/dynorphin neurons of the arcuate nucleus; E_2: estradiol; ER: estrogen receptor α; OC: optic chiasm; PMR: premamillary region; RCh: retrochiasmatic nucleus; vmPOA: ventromedial preoptic area.

positive neurons expressed Fos, a marker for neuronal activation, when exposed to E_2 in anestrous only; thus, confirming that these dopaminergic neurons are responsive to seasonal E_2 negative feedback (Lehman *et al.* 1996). In sum, there is strong evidence implicating the dopaminergic cells of the A15 region in conveying the inhibitory influence of E_2 to GnRH neurons during anestrous.

1.5.2 Circuitry upstream of the A15 cell group

ERα is the steroid receptor implicated conveying the upstream influence of E_2 onto A15 neurons, as a selective ERβ agonist injected into the retrochiasmatic region failed to alter LH concentration in ovariectomized ewes (Hardy *et al.* 2003). Since, like GnRH neurons, A15 dopaminergic neurons do not express ERα, the influence of E_2 is likely to be conveyed from another cell population (Lehman and Karsch, 1993). Tract tracing studies found four candidate regions that both project to the A15 dopaminergic neurons and contain cells that express ERα: ventromedial preoptic area (vmPOA), retrochiasmatic area (RCh), arcuate nucleus and ventromedial hypothalamic nucleus (Coolen *et al.* 1999). Neurons in the vmPOA are strong candidates for the E_2 negative feedback pathway as this region contains ERα positive cells that express Fos in response to low circulating levels of E_2 during anestrous but not the breeding season (Stefanovic *et al.* 2000). Further experiments confirmed the that these ERα containing neurons from the vmPOA project to A15 dopaminergic cells (Lehman 2002) and that E_2 microimplants into the vmPOA in ovariectomized anestrous ewes can inhibit GnRH secretion (Anderson *et al.* 2001). Strong evidence also implicates the ERα expressing cell population in the RCh in the E_2 negative feedback system as E_2 microimplants in ovariectomized anestrous ewes in this region inhibited LH pulses, an effect that was blocked by dopamine antagonist sulpiride

(Hardy *et al.* 2003). Thus, it is hypothesized that E_2 enters the negative feedback pathway by acting on cells in either the vmPOA or RCh.

A recent study by Bogusz *et al.* (2008) found strong evidence supporting the role of γ-aminobutyric acid (GABA) as the neurotransmitter involved in transferring the inhibitory influence of E_2 in anestrous to the A15 region. Specifically, $GABA_A$ and $GABA_B$ receptor agonists injected into the A15 increased LH secretion. Furthermore, A15 dopaminergic neurons expressed $GABA_A$ (30%) and $GABA_B$ (nearly all) receptors and were contacted by glutamic acid decarboxylase (GAD: a marker for GABA)-containing axon terminals. However, the number of GABA contacts or receptors did not change with season and thus, while GABAergic neurons may be part of the circuit, changes in their input to A15 neurons are unlikely to be responsible for the seasonal variation in the ability of E_2 to inhibit GnRH secretion. Other neurotransmitter inputs may also play a role, as a two-fold increase in the number of synaptic inputs onto A15 dopaminergic dendrites was observed in anestrous (Adams *et al.* 2006). Whether GABAergic cells that provide input to A15 are the same population that express ERα in vmPOA or RCh remains to be determined. To sum, low levels of circulating E_2 found in anestrous are hypothesized to inhibit GABAergic cells in the vmPOA or RCh which release inhibition of A15 dopaminergic neurons ultimately decreasing LH pulse frequency.

1.5.3 Circuitry downstream of the A15 cell group

Whether the influence of dopamine is conveyed directly from the A15 cell group to GnRH neurons, or indirectly via interneurons, is not known. A15 neurons have been shown to project to the median eminence as well as sparsely to the mediobasal hypothalamus (MBH) (Lehman 2002). There is evidence that GnRH neurons in the ovine MBH (as well as POA) receive tyrosine hydroxylase (TH: enzyme used to detect catecholaminergic neurons)-positive inputs

(Jansen *et al.* 2003), as well as evidence for axo-axonic contacts between dopamine and GnRH fibres in the median eminence (Kuljis and Advis 1989); however the number of contacts at both the level of the GnRH cell body and terminal is low. Further, a third of TH contacts onto GnRH cells in the POA were not dopaminergic as they were also immunoreactive for the enzyme dopamine β-hydroxylase (DBH: a marker for epinephrine or norepinephrine neurons) (Pompolo *et al.* 2003). Both TH-positive contacts (Jansen *et al.* 2003) and DBH-positive contacts (Pompolo *et al.* 2003) do not vary with season suggesting they are not related to the control of seasonal reproduction. Due to the paucity of dopaminergic contacts and the abundance of other contacts onto GnRH cell bodies it is thus likely that there are interneurons between the A15 region and GnRH. Nevertheless it is important to determine if GnRH cells in the MBH express dopamine receptor D2 (D2R), the receptor implicated in E_2 negative feedback (Curlewis *et al.* 1991), as this would support the hypothesis that A15 neurons act directly upon MBH GnRH neurons.

Pharmacological studies examining the effect of dopamine D1 and D2 receptor (D2R) specific agonists and antagonists on pulsatile LH secretion implicated D2R as the receptor involved in E_2 negative feedback (Curlewis *et al.* 1991). D2R is a seven transmembrane G-protein coupled receptor that inhibits adenylyl cyclase, downregulating the cAMP–dependent pathway. D2R can also link to intracellular calcium stores and ion channels via Gβγ subunits, as well as act via protein kinase B (Akt)-GSK-3 signalling cascade. Combined, these pathways allow D2R to influence voltage gated ion channels, ligand gated glutamate receptors and ion pumps. Like many G-protein coupled receptors, it is internalized upon activation (Yao *et al.* 2008). Specifically, D2R is rapidly phosphorylated by GRKs (G protein-coupled receptor kinases) upon being activated by ligands and binds to arrestins which target the receptor for clatharin-mediated endocytosis (for review of aforementioned D2R pathways and internalization see Yao *et al.* 2008). Internalization can thus be used as a marker for activation (Namkung *et al.* 2009).

D2R has two isoforms that are thought to have different roles. The short form receptor is primarily a presynaptic autoreceptor whereas the long form receptor, which contains an additional 29 amino acids, is mainly postsynaptic (Usiello *et al.* 2000). Recent pharmacological manipulations have found that E_2 may be involved in regulating the expression of D2R themselves as the D2R agonist quinpirole failed to reduce LH secretion in anestrous ewes that were ovariectomized (Goodman 2009). Localizing D2R in cell populations hypothesized to lie downstream of the A15 in the E_2 negative feedback pathway will provide evidence of potential sites of action for the inhibitory influence of A15 dopamine.

1.5.4 Potential interneurons downstream of the A15 cell group

There are a number of potential interneurons that may convey the inhibitory influence of A15 dopamine onto GnRH neurons as the latter receive a diverse array of synaptic inputs. It is possible that plasticity in these synaptic inputs is responsible for the dramatic increase in sensitivity of the GnRH neuron to E_2 negative feedback in anestrous. Ovine GnRH neurons have been shown to exhibit seasonal plasticity in their number of synaptic inputs, receiving more than twice the density of synapses in the breeding season compared to anestrous (Xiong *et al.* 1997). Complementing this finding was the discovery that polysialylated form of neural cell adhesion molecule (PSA-NCAM), a support molecule for axonal guidance and synaptogenesis, was significantly increased along the surface of GnRH neurons in breeding season compared to anestrous (Viguié *et al.* 2001). While the direction of the change in synaptic density onto GnRH neurons (breeding season > anestrous) is opposite to what one might expect given the increase in responsiveness to E_2 negative feedback (anestrous > breeding season), a higher number of stimulatory synaptic inputs per GnRH neuron predominate during the breeding season compared to anestrous (Jansen *et al.* 2003). Thus, the balance of stimulatory *vs.* inhibitory inputs

to GnRH neurons in the ewe appears to change seasonally, with a greater proportion of inhibitory inputs during anestrous. This has led to speculation that synaptic plasticity in presynaptic contacts onto GnRH neurons may contribute to seasonal variation in responsiveness of GnRH neurons to the negative feedback influence of E_2. However, it is unlikely that the variation in synaptic input at the level of GnRH cells is solely responsible for seasonal changes in responsiveness to E_2 negative feedback system since thyroidectomized ewes, in which seasonal rhythms are disrupted and which remain in breeding condition, have the same pattern of synaptic input onto GnRH cells as do intact anestrous ewes (Adams *et al.* 2006).

A strong possible candidate interneuron for conveying the influence of A15 dopamine neurons on GnRH cells are a subpopulation of neurons in the arcuate nucleus that co-express three neuropeptides kisspeptin, neurokinin B, and dynorphin (termed "KNDy" cells), and which have recently been shown to play a pivotal role in steroid feedback control of GnRH secretion (Goodman *et al.* 2007). The KNDy cell population is a major potential target for the feedback actions of ovarian steroids, since virtually all of these cells express both ERα as well as progesterone receptors (Franschini *et al.* 2006, Foradori *et al.* 2002). Two of the KNDy cells peptides, kisspeptin and neurokinin B (NKB), have been the center of intense research focus over the past few years; both have been implicated as important for normal reproductive function in animal models (Roa *et al.* 2009, Sandoval-Guzmán and Rance 2004), as well as in humans, where mutations in either kisspeptin or NKB, or their receptors, lead to hypogonadotropic hypogonadism (Roseweir and Millar 2009, Topaloglu *et al.* 2009). In sheep, kisspeptin mRNA and peptide in KNDy cells, as well as the number of kisspeptin contacts onto GnRH cells, have been shown to vary seasonally and parallel seasonal changes in responsiveness to E_2 negative feedback (Smith *et al.* 2008). Importantly, kisspeptin mRNA and peptide expression is decreased during anestrous (Smith *et al.* 2008); since kisspeptin is exceptionally

stimulatory to GnRH release (Messager *et al.* 2005), one possibility is that dopamine from the A15 region inhibits GnRH release indirectly, by inhibiting KNDy cells and thereby the release of kisspeptin. One way to test this possibility is to determine if KNDy cells express dopamine receptor D2 (D2R), as this would support the hypothesis that A15 neurons act via these interneurons to inhibit GnRH secretion.

Two additional potential candidate interneurons for relaying the inhibitory influence of A15 dopamine cells onto GnRH neurons are gonadotropin-inhibitory hormone (GnIH) and orphanin FQ (OFQ)-expressing neurons. GnIH cell bodies are located in the dorsomedial hypothalamic nucleus and appear to directly contact GnRH neurons (Smith *et al.* 2008). Like kisspeptin, GnIH shows seasonal changes in its expression, but is increased during anestrous at the same time that kisspeptin mRNA and peptide is decreased (Smith *et al.* 2008). The reciprocal relationship between kisspeptin and GnIH has led to the hypothesis that these two peptides serve as a balance of stimulatory and inhibitory drive to GnRH neurons, respectively, and thereby contribute to seasonal shifts in GnRH secretion (Kriegsfeld 2006). The possibility that A15 dopamine cells may regulate GnRH secretion via changes in GnIH expression has not yet been explored. OFQ is an opioid family peptide, which is expressed both in GnRH cells as well as in a separate group of neurons in the arcuate nucleus (Foradori *et al.* 2007). OFQ has been shown to play a role in the inhibitory control of GnRH pulses *in vivo* (Foradori *et al.* 2007), but its role in conveying the inhibitory influence of dopamine from the A15 to GnRH cells has also not been explored.

1.6 Summary

In summary, although significant progress has been made towards understanding the neural circuitry responsible for the reversible suppression of GnRH secretion, much remains to

be discovered. The molecular and/or morphological changes in this circuitry that are responsible for the dramatic increase in sensitivity of GnRH to the negative influence of E_2 during anestrous remain to be elucidated. In this thesis, I have carried out studies to identify potential site(s) of action where dopamine may act to inhibit GnRH pulses; specifically, I hypothesized that A15 dopaminergic neurons influence GnRH secretion either directly by synaptic contact on GnRH neurons themselves, or indirectly via inputs to KNDy interneurons. I examined these possibilities by asking whether D2R are colocalized in either GnRH and/or KNDy neurons, using dual-label immunocytochemistry (Figure 3). In addition, I performed seasonal comparisons to determine whether changes in D2R expression in either cell population may contribute to seasonal differences in the ability of E_2 to inhibit GnRH pulses. I then explored the possibility that internalization of the D2R receptor in GnRH or KNDy cells could be used as a marker of endogenous release of dopamine *in vivo*. Finally, I obtained evidence that kisspeptin terminals provide direct synaptic input to GnRH neurons, supporting the possibility that KNDy cells act as an intermediary for the effects of dopamine on GnRH secretion. The results revealed both GnRH and KNDy cell populations as potential sites of action where A15 dopamine could potentially act to regulate GnRH secretion, but suggested that KNDy cells, in particular, may be particularly well positioned to play an important role in the circuitry underlying the shifts in E_2 negative feedback that are responsible for seasonal reproductive transitions.

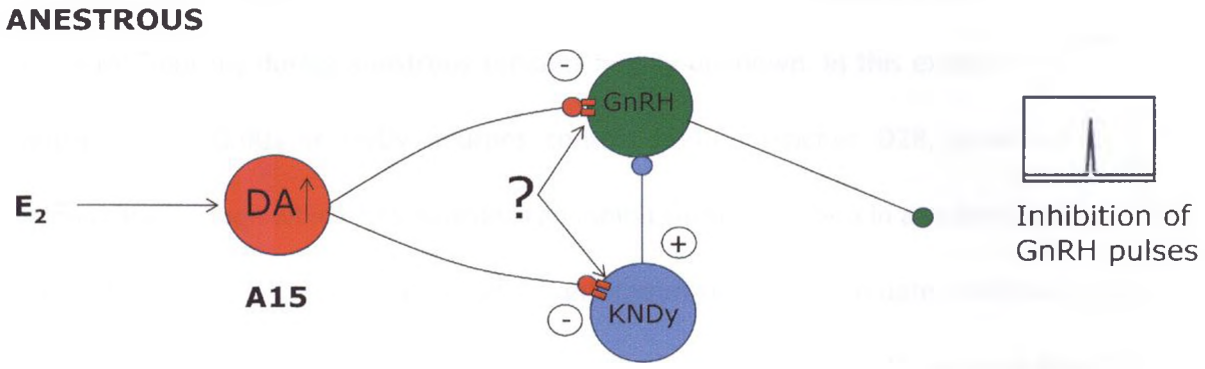


Figure 3 Potential site(s) of action where dopamine may act to inhibit GnRH pulses. Hypothesized pathways by which A15 neurons can inhibit GnRH secretion in anestrous are explored in this thesis by determining if D2R are colocalized in either GnRH and/or KNDy neurons, using dual-label immunocytochemistry.

Chapter 2

The localization of dopamine D2 receptors in the neural circuitry underlying seasonal reproduction in the sheep

2.1 Experiment 1: Seasonal changes in D2R colocalization in GnRH and KNDy cells

2.1.1 Rationale

The mechanism by which A15 dopaminergic neurons convey the inhibitory influence of E_2 to GnRH neurons during anestrous remains largely unknown. In this experiment I tested the hypothesis that GnRH or KNDy neurons contain immunoreactive D2R, providing a possible intermediary through which A15 neurons can inhibit GnRH secretion in anestrous, either directly via GnRH neurons or indirectly via KNDy interneurons in the arcuate nucleus. Specifically, expression of D2R in GnRH cells would support the hypothesis that A15 neurons directly inhibit GnRH secretion via dendritic or somatic contacts, in addition to previously described axonal contacts in the median eminence (Kuljis and Advis, 1989). Expression of D2R in KNDy cells would support the role of this cell population as an important mediator of the inhibitory influence of dopamine in anestrous. In addition, since A15 neurons are known to project caudally from the retrochiasmatic area to the mediobasal hypothalamus (MBH), a regional difference in D2R expression among GnRH cells, with a greater percentage of colocalization in GnRH cells located in the MBH rather than the preoptic area, would be consistent with potential inputs from A15 dopamine cells. I also examined possible seasonal variations in the expression of D2R in GnRH and KNDy neurons. Previous experiments have shown that E_2 activates A15 dopaminergic neurons only in anestrous (Lehman *et al.* 1996). Consequently, D2R expression in either cell population might be expected to be highest in anestrous and decrease when the animal enters the breeding season.

Dual-label fluorescent immunocytochemistry was employed to detect the expression of D2R in either GnRH or KNDy cells. Since kisspeptin mRNA and peptide is decreased in the arcuate nucleus of anestrous ewes (Smith *et al.* 2008), I could not use this as a marker for KNDy cells, and instead used the co-expressed neuropeptide, dynorphin, as a marker since this peptide does not show seasonal variation (Lehman, unpublished observations). Dynorphin is also present in a small number of cells in the preoptic area, but unlike the KNDy cells in the arcuate nucleus, these do not colocalize kisspeptin or neurokinin B (Goodman *et al.* 2007). Sections for dual-label D2R/GnRH and D2R/dynorphin were processed in both anestrous and breeding season ewes for regional and seasonal comparisons. The intracellular localization of D2R in GnRH and dynorphin cells was also qualitatively assessed as this G-protein coupled receptor becomes internalized upon activation. I expected an increase in the degree of receptor internalization in either cell population in anestrous given the increased endogenous dopamine release from axons arising from A15 cells at this time of year.

2.1.2 Methods

Animals and tissue collection

Mature Suffolk black-faced ewes (*Ovis aries*) were maintained indoors under natural photoperiod with free access to water and a maintenance diet at the West Virginia University Research Farm. Ewes were ovariectomized (OVX) and given subcutaneous Silastic E_2 implants to maintain constant, low physiological levels of circulating E_2. In this well characterized model of seasonality in the ewe (Lehman *et al.* 1997), seasonal changes in responsiveness to E_2 can be easily monitored by assaying circulating levels of luteinizing hormone (LH); LH remains high during the breeding season and decreases to undetectable levels in anestrous. Animals were sacrificed either in anestrous (n= 5) or breeding season (n = 5) by an injection of heparin

followed by an overdose of sodium pentobarbital. Animals were immediately decapitated and the heads perfused via both internal carotid arteries with 6L of 4% paraformaldehyde in 0.1M phosphate buffer (PB) containing 0.1% $NaNO_3$.

Blocks containing the preoptic area and hypothalamus were dissected from fixed brains and soaked in 4% paraformaldehyde overnight at 4°C. Tissue blocks were then stored in a 30% sucrose-PB solution at 4°C. Tissue blocks were sliced into six series of coronal sections 45µm thick using a freezing microtome (Microm HM 400). Tissue sections were subsequently stored at -20°C in cryoprotectant solution (0.004% Sodium Azide, 30% ethane-1, 2-diol, and 300g sucrose per L in 0.1M PB). One series per animal was processed for dual immunocytochemical detection of D2R and GnRH. A second series per animal was processed for immunocytochemical detection of D2R and dynorphin.

Western blotting

A hypothalamic block from an ovary-intact anestrous ewe was immediately frozen upon dissection and stored at -80°C. A sample of the arcuate nucleus was obtained using a micropunch technique and homogenized mechanically in Radio Immuno Precipitation Assay (RIPA) buffer (50mM Tris-HCl, 150mM NaCl, 1% Nonidet P 40, 0.1% SDS, 0.5% sodium deoxycholate in ddH_2O) with a protease inhibitor cocktail tablet (Hoffman-La Roche Ltd.). The homogenization mixture was centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant collected. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (ThermoFisher Scientific Inc.) and a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific Inc.). Protein from a human whole cell lysate (sc-2410, Santa Cruz Biotechnology) was also processed as a positive control. Protein was heated at 96°C for 4 minutes and cooled on ice for 2 minutes. Gels containing a 5mL of stacking gel (833 µL Acrylamide, 1.25 mL 0.5M tris (pH

6.8), 50 µL 10% SDS, 50 µL 10% APS, 5 µL TEMED in 2.9 mL of ddH_2O) and 20mL of separating gel (6.7µL Acrylamide, 5 mL 0.5M tris (pH 6.8), 200 µL 10% SDS, 200 µL 10% APS, 15 µL TEMED) were loaded with protein samples in SDS-PAGE Western blotting buffer and separated using gel electrophoresis at 85V until the maker reached the preset level on the Mini-PROTEAN Tetra cell (Bio-Rad). Gels were washed and an Immobilon-FL Transfer Membrane (PVDF) (previously soaked in transfer buffer for 15 seconds and activated with methanol) was added to the surface. In transfer buffer cooled with ice, protein was transferred to the transfer membrane by electroblotting (1 hour at 85V). Protein was visualized by first blocking the transfer membrane with 60% TBS (60.5g of 0.5M Tris and 87.6g of 1.5M NaCl per L at a pH of 8.0 diluted 1:10 in ddH_20) and 40% Odyssey blocking buffer (Li-Cor) for 30 minutes. Transfer membrane was then incubated for 17 hours with the primary antibody mouse anti-D2R (Santa Cruz Biotechnology) at 1:500, 1:1000, 1:2000 in 60% TBST (1xTBS with 0.05% tween-20 pH of 7.4) and 40% Odyssey blocking buffer (Li-Cor). After washing in TBST for 10 minutes three times, the transfer membrane was incubated with the secondary antibody IRDye 680 Goat Anti-Mouse (Li-Cor) at a concentration of 1:5000 in TBST for one hour. Transfer membrane was washed and scanned with the Odyssey infrared imaging system (Li-cor).

Immunocytochemistry

Free-floating tissue sections were washed of cryoprotectant in 0.1M PBS for 3 hours in 15 minute intervals. Tissue sections were then blocked in 1% H_2O_2 in 0.1M PBS for 10 minutes and rinsed in 0.1M PBS 4 times for 5 minutes each. Sections were then rinsed for one hour in PBS+ (0.1M PBS with 4% Triton-X and 4% normal goat serum), and subsequently incubated with mouse anti-D2R antibody (Santa Cruz Biotechnology) at 1:100 in PBS+ for 17 hours.

Tissue sections were then rinsed (0.1M PBS for 5 minutes, 4 times) and rinsed again following each subsequent step. Next, the sections were incubated in the secondary antibody, biotinylated goat anti-mouse (Vector Laboratories) at 1:500 in PBS+ for 1 hour. To amplify the signal, sections were first incubated in ABC-elite (VECTASTAIN ABC kits, Vector laboratories) 1:500 in PBS for 1 hour. Next, the signal was amplified using the tyramide signal amplification system by incubating sections with biotinylated tyramine (PerkinElmer) at 1:250 in PBS containing 0.003 % H_2O_2 for 10 minutes. Tissue sections were then incubated for 30 minutes in Alexa 488 Fluor conjugated streptavidin (Molecular Probes) 1:100 in PBS.

For the first portion of the experiment, the tissue sections were then incubated in mouse anti-GnRH (Sternberger) at 1:500 in PBS+ for 17 hours. The sections were subsequently incubated with the secondary antibody Alexa Fluor 555 goat anti-mouse (Molecular Probes) 1:100 in PBS (with 4% Triton-X) for 30 minutes. For the second portion of the experiment, tissue sections were incubated with rabbit anti-dynorphin A (Phoenix) antiserum in PBS+ for 17 hours. As in the first experiment, post-rinsing, the sections were incubated for 30 minutes in the secondary antibody Alexa Fluor 555 goat anti-rabbit (Molecular Probes) 1:100 in PBS (with 4% Triton-X). In both cases, following processing, the tissue sections were mounted on glass slides using 0.3% gelatin and coverslipped using gelvatol. Slides were stored in the dark at 4°C.

Controls for immunocytochemistry included the omission of each primary antibody from the protocol described above, as well as the Western blot described above to confirm specificity of the D2R antibody. In addition, preabsorption controls, in which the diluted primary antiserum is incubated for 24 h at 4°C with nanomolar concentrations of corresponding purified antigen, have been previously conducted for both mouse anti-GnRH (Sternberger) and rabbit anti-dynorphin A (Phoenix) by our lab (Goodman *et al.* 2004, Goodman *et al.* 2007).

Data Analysis

The number of single- and double-labelled GnRH neurons were counted in every section processed through the preoptic area (POA), anterior hypothalamic area (AHA) and mediobasal hypothalamus (MBH) of each animal (anestrous n = 5, breeding season n = 5) using a Leica DM5000B fluorescence microscope at 20x and 40x magnification (Leica Microsystems). The percentage of GnRH neurons colocalizing D2R in each region was calculated for each animal. The mean percentage of GnRH cells colocalizing D2R was then determined for each region in each season. Similarly, the number of single- and double-labelled dynorphin neurons were counted throughout every section processed in the POA and in two sections of sampled from both the middle, and caudal levels of the arcuate nucleus of each animal (anestrous n = 5, breeding season n = 5) using a Leica DM5000B fluorescent microscope (Leica Microsystems) with a motorized stage (MBF Biosciences) at 20x magnification. Because of the density of dynorphin cells in the arcuate nucleus, Neurolucida software (MBF Biosciences) was used to append multiple 2-channel images. Only dynorphin cells with a clear nucleus present at the focal plane of the image were counted. The percentage of dynorphin neurons colocalizing D2R in each region was calculated for each animal. The mean percentage of dynorphin cells colocalizing D2R was then determined for each region in each season. In addition, as a control, in each animal I analysed the number of single- and doubled-labelled dynorphin cells in two sections of the supraoptic nucleus (SON) of the hypothalamus in each animal; dynorphin cells are found in the SON, but there is no evidence for seasonal or steroid-induced changes in this population (Foradori *et al.* 2005). The percentage of dynorphin neurons colocalizing D2R in this region was calculated for each animal and the mean percentage was then determined for each season.

To examine the intracellular position of D2R, confocal Z-stacks of at least 10 GnRH cells colocalizing D2R were taken from sections of both the POA and MBH in anestrous and breeding

season ewes. Similarly, confocal Z-stacks of at least 10 dynorphin cells colocalizing D2R were taken from sections of the middle arcuate nucleus in both anestrous and breeding season ewes. Confocal images were acquired at 63x magnification using a LSM 510 META/ConfoCor2 (Carl Zeiss, Inc) with LSM-510 META image processing software. A 543nm emission filter was used on a HeNe laser to view Alexa 555 fluorescent staining. A 488nm emission filter was used on an Argon laser to visualize the Alexa-488 fluorescent staining. Z-stacks were produced with images captured at 1μm intervals through the entire neuron (~20 μm).

Statistical comparisons were made in the number of GnRH cells analyzed in each region between seasons using unpaired student's t-tests. A two-way ANOVA was used to determine if there were any regional or seasonal differences in percent colocalization of D2R in GnRH cells. In addition, statistical comparisons were made in the number of dynorphin cells analyzed in each region using unpaired student's t-tests. A two-way ANOVA with Tukey's Honestly Significant Differences post-hoc test was used to determine if there were any regional or seasonal differences in percent colocalization of D2R in dynorphin cells of the POA, middle and caudal arcuate nucleus. Seasonal comparison of the percentage of dynorphin cells coexpressing D2R in the SON control region was examined using an unpaired student's t-test. All data was examined for equal variance using Bartlett test of homogeneity of variances and normal distribution using the Shapiro-Wilk normality test. Differences between means were considered statistically significant if the p value obtained was less than 0.05.

2.1.3 Results

Controls for immunocytochemistry

A western blot was used to confirm the specificity of the mouse anti-D2R antibody (Santa Cruz Biotechnology) (B-10): sc-5303 (Figure 4). A band was present in both the control cell

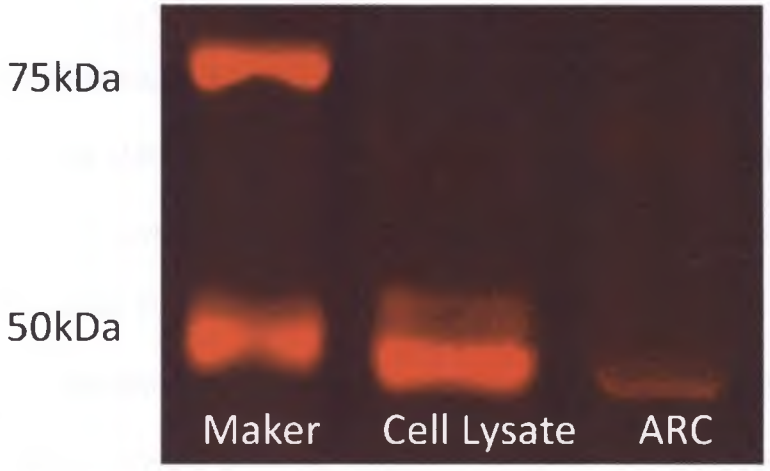


Figure 4 Western blot of dopamine D2 receptor. A single immunoreactive band is present at the appropriate weight in both the positive control human whole cell lysate and a protein sample taken from the arcuate nucleus (ARC) of the ovine hypothalamus. Molecular weights of the short and long forms of D2R are 48 and 51 kDa.

lysate and protein from the ovine arcuate nucleus at the correct molecular weight corresponding to D2R. The intensity of immunoreactivity decreased with dilution of the antibody from 1:500 to 1:2000 (data not shown). As described above, immunocytochemical controls for the dual-label procedure included omission of each primary antibody separately. Omission of D2R antibody eliminated all D2R staining with dynorphin immunoreactivity remaining unaltered. Conversely, omission of dynorphin primary antibody eliminated the dynorphin signal but the D2R signal remained intact.

D2R colocalization in GnRH cells

Distinct D2R immunoreactivity was present as punctate immunoreactivity in GnRH cells in the POA, AHA, and MBH in ewes sacrificed during anestrous and breeding season. The total number of GnRH cells detected between seasons did not vary (Figure 5). The percentage of GnRH cells colocalizing D2R did not differ significantly between brain regions or between seasons (Figure 6). Bartlett tests of homogeneity of variances found no significant differences in variance in the data analyzed. Likewise, Shapiro-Wilk normality tests determined the data was normally distributed. No differential subcellular localization of receptor was evident as nearly all D2R immunoreactive particles appear to be cytoplasmic in GnRH cells regardless of region or season (Figure 7).

D2R colocalization in KNDy cells

D2R immunoreactivity was present in dynorphin cells in the middle and caudal levels of the arcuate nucleus in ewes sacrificed during anestrous and breeding season. The total number of dynorphin cells detected did not vary between seasons in any region examined (Figure 8). Bartlett tests of homogeneity of variances found no significant difference in variances in the percentage of dynorphin cells expressing D2R by season but did find a regional difference.

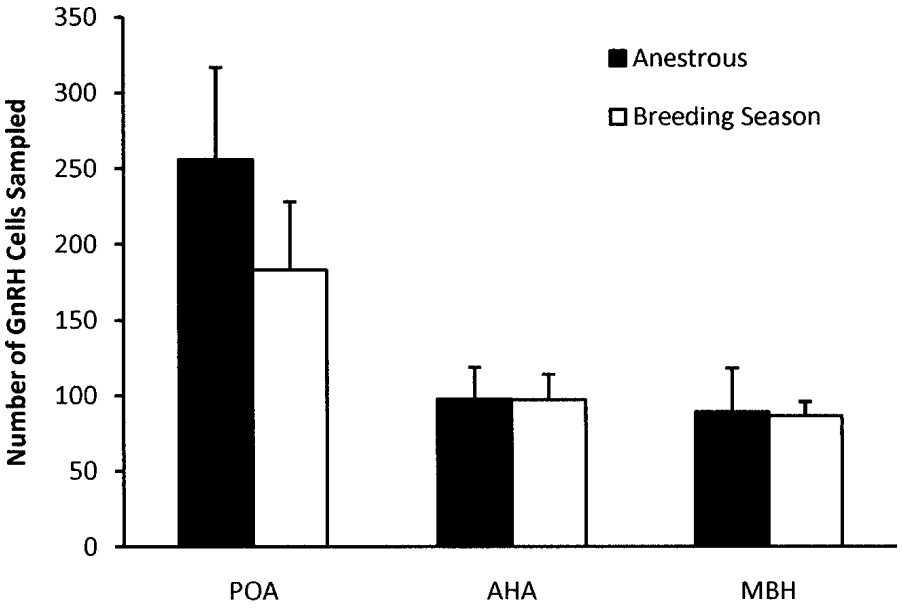


Figure 5 Total number of GnRH cells (mean ± SEM) in the preoptic area (POA), anterior hypothalamus (AHA) and mediobasal hypothalamus (MBH) analysed in anestrous and breeding season. No significant differences were found using unpaired student's t-tests in the number of GnRH cells counted between seasons in any region examined (anestrous n = 5, breeding season n = 5).

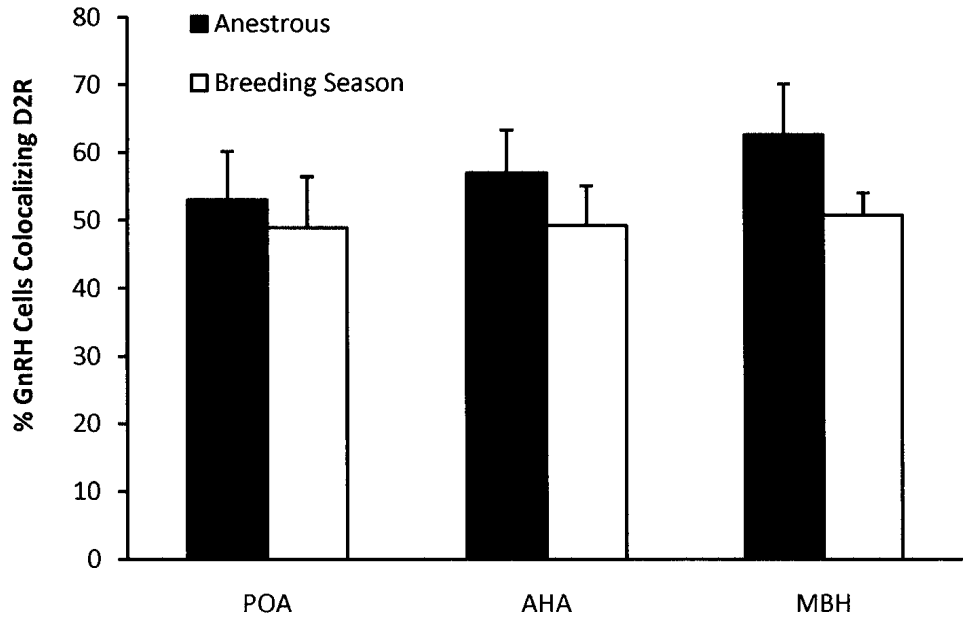


Figure 6 Percent colocalization of D2R and GnRH (mean ± SEM) in the preoptic area (POA), anterior hypothalamus (AHA) and mediobasal hypothalamus (MBH), analysed in anestrous and breeding season. No significant differences were found using a two-way ANOVA in the coexpression of GnRH and D2R between seasons or between regions (anestrous n = 5, breeding season n = 5).

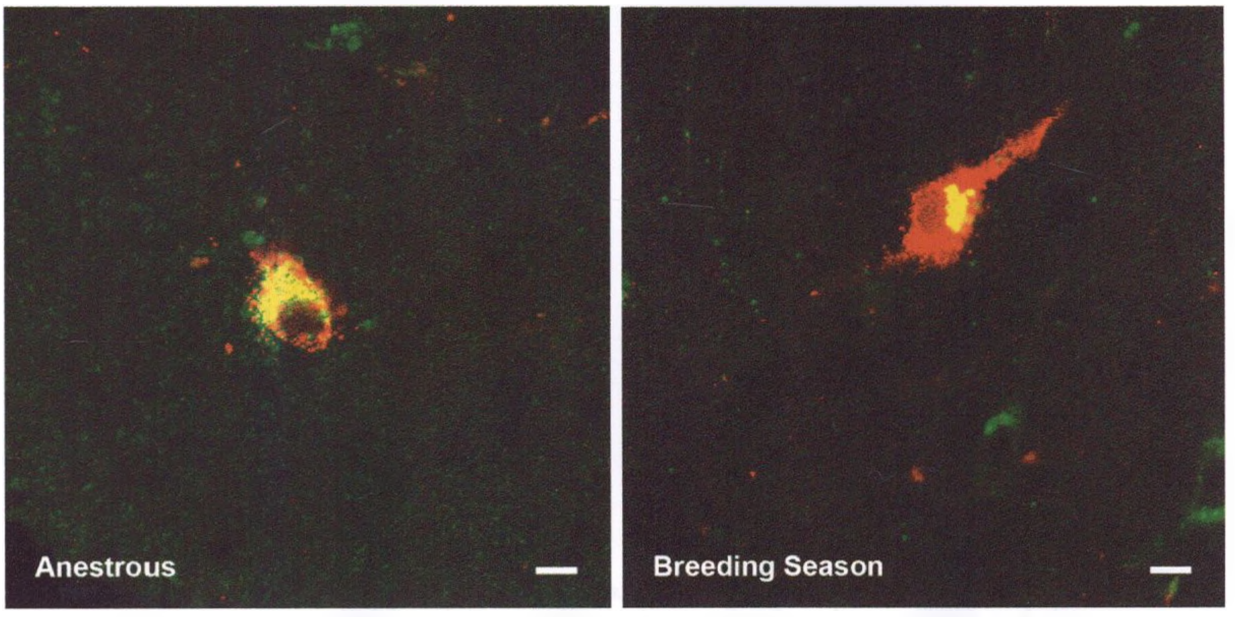


Figure 7 Confocal images of examples of D2R and GnRH coexpression in anestrous and breeding season ewes. Dual-label immunofluorescent confocal images (1 µm optical section) of representative GnRH neurons from the MBH of ewes in anestrous and breeding season. D2R immunoreactivity is in green, GnRH in red; colocalization is indicated by patches of yellow. *Scale bar*, 10 µm.

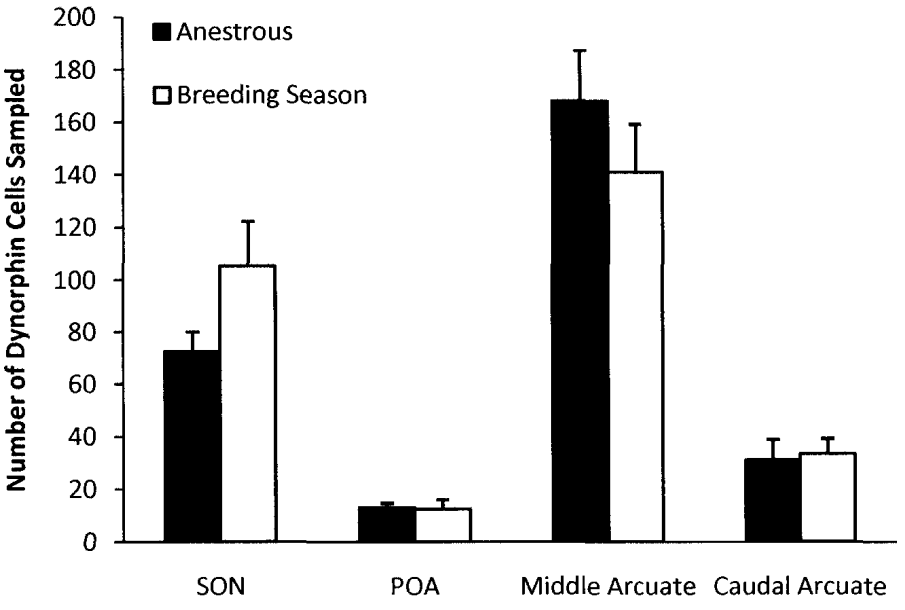


Figure 8 Total number of dynorphin cells (mean ± SEM) in the supraoptic nucleus (SON), preoptic area (POA), middle and caudal arcuate nucleus, analysed in anestrous and breeding season ewes. No significant differences were found using unpaired student's t-tests in the number of dynorphin cells counted between seasons in any region examined (anestrous n = 4, breeding season n = 5).

Shapiro-Wilk normality tests determined the data was normally distributed. A two-way ANOVA was used even though the variances in the data between regions were not equal as this test is generally robust to such failed assumptions. The percentage of dynorphin cells expressing D2R in the POA was very low (<10%) and significantly less than in that seen in either the arcuate KNDy subpopulation or the SON, regardless of season (Figure 9).

Seasonal differences in the percentage of dynorphin cells colocalizing D2R were seen in the arcuate nucleus but not in the POA nor in the SON (Figure 9, Figure 10). Specifically, the percentage of dynorphin cells colocalizing D2R was significantly reduced by 30-40% in the middle and caudal ARC of breeding season compared to anestrous ewes (Figures 9, 11). In addition, I noted that there appeared to be greater numbers of non-dynorphin, D2R-immunolabeled cells in the ARC of anestrous ewes (Figure 11) although I did not quantitatively analyze the number of single-labelled D2R cells. As was found in GnRH cells, no obvious seasonal or regional differences in subcellular localization as almost all D2R immunoreactivity appeared to be cytoplasmic, in dynorphin cells as well as other adjacent neurons (Figure 10).

2.1.4 Conclusions

The colocalization of dopamine D2 receptors in at least 50% of GnRH neurons indicates that it is possible for dopamine to interact monosynaptically with GnRH cell bodies in addition to GnRH terminals in the median eminence where dopaminergic contacts have previously been identified (Kuljis and Advis, 1989). Earlier studies also found evidence for TH-positive contacts onto GnRH cell bodies and dendrites (Lehman *et al.* 1987), however these are very low in number (Jansen *et al.* 2003). As there is no regional difference in D2R colocalization between GnRH cells in the MBH, where A15 neurons project, and GnRH cells in the POA, it seems unlikely that this receptor in GnRH cells is positioned to respond specifically to A15 input. In addition, the

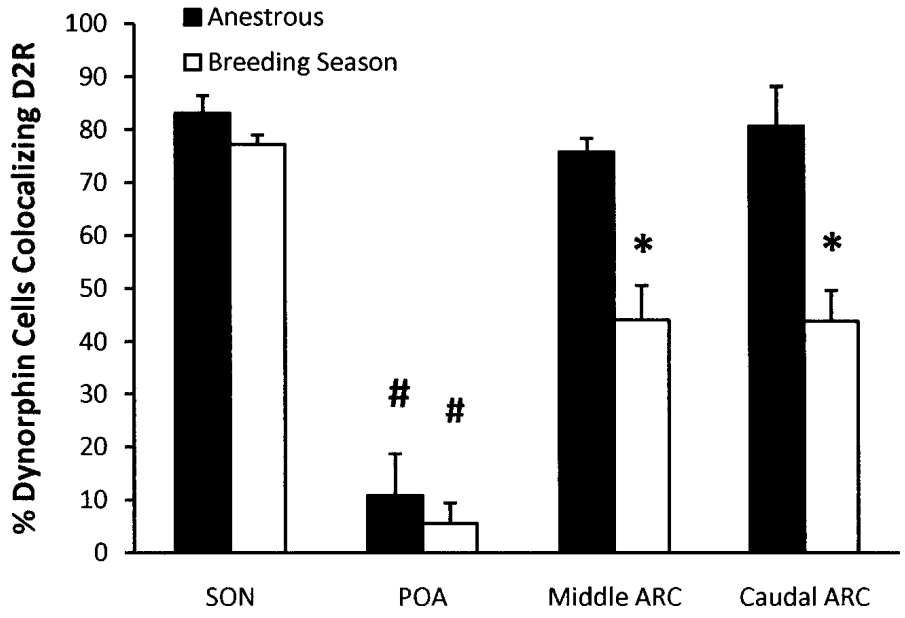


Figure 9 Percent colocalization of D2R and dynorphin (mean ± SEM) in the supraoptic nucleus (SON), preoptic area (POA), middle and caudal arcuate nucleus of anestrous and breeding season ewes. Significant seasonal differences in coexpression of dynorphin and D2R were found using a two-way ANOVA with a Tukey's Honestly Significant Differences post-hoc test in the POA and arcuate nucleus (seasonal: $F_{(1,19)} = 46.689$, $p < 0.0001$, regional: $F_{(2,19)} = 153.744$, $p < 0.0001$, interaction: $F_{(2,19)} = 13.038$, $p = 0.00027$). Specifically, significant differences between seasons in the percent of dynorphin cells colocalizing D2R in the middle arcuate ($p = 0.00021$) and caudal arcuate ($p < 0.00002$) but not the POA. Significantly fewer cells colocalized D2R in the POA compared to the middle arcuate (anestrous $p < 0.0001$, breeding season $p < 0.0001$) and caudal arcuate (anestrous $p < 0.0001$, breeding season $p < 0.0001$) but there were no differences between regions of the arcuate. No significant difference was found using an unpaired student's t-test on the SON control region. *, $p < 0.01$ anestrous *vs.* breeding season. #, $p < 0.01$ POA *vs.* both middle arcuate and caudal arcuate (anestrous $n = 4$, breeding season $n = 5$).

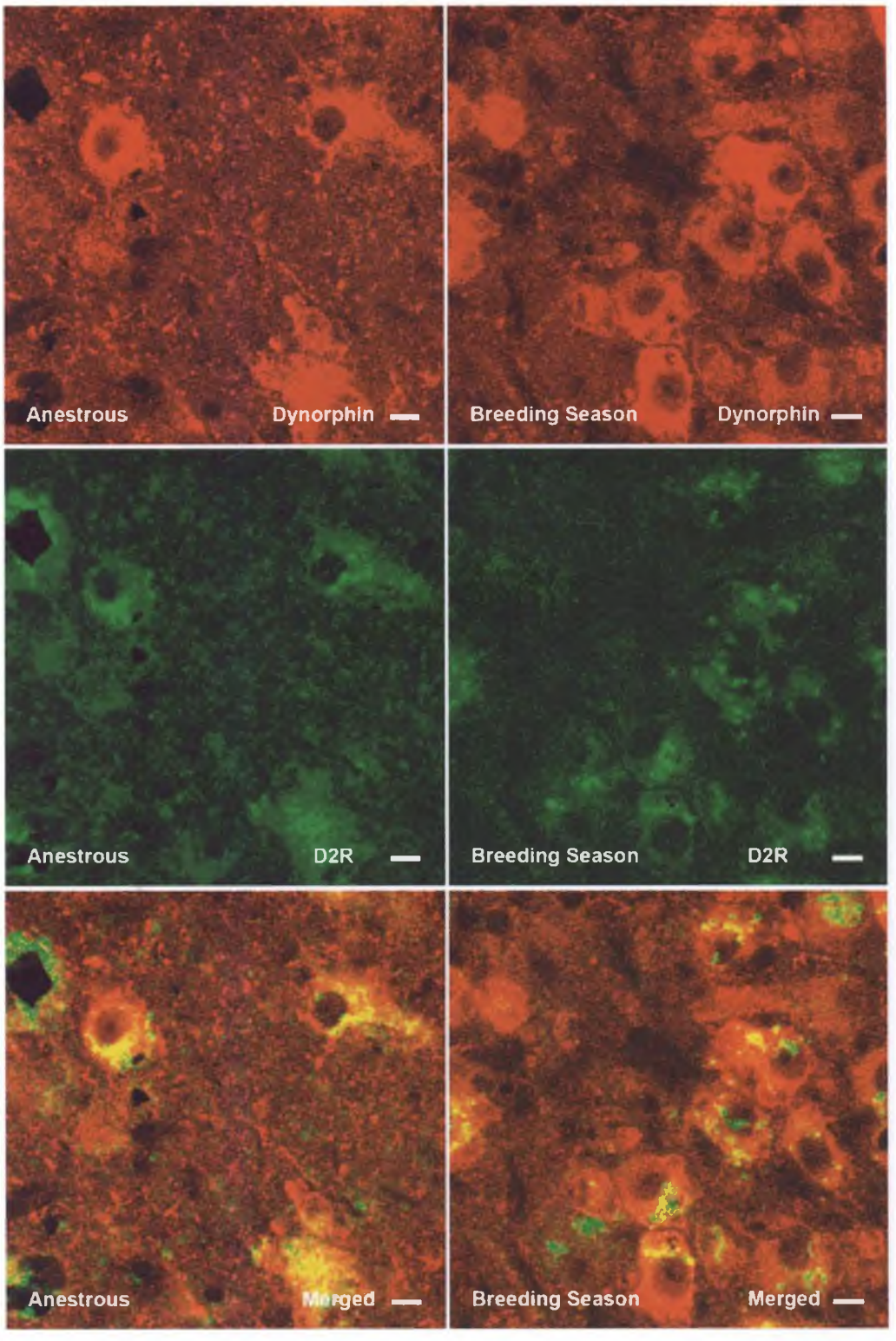


Figure 10 Confocal images of examples of D2R and dynorphin colocalization in the supraoptic nucleus (SON) of anestrous and breeding season ewes. Dual-label immunofluorescent confocal images (1 µm optical section) of representative dynorphin neurons from the SON of ewes in anestrous and breeding season. D2R immunoreactivity is in green, dynorphin in red; colocalization of the signals is shown by yellow. *Scale bar*, 10 µm

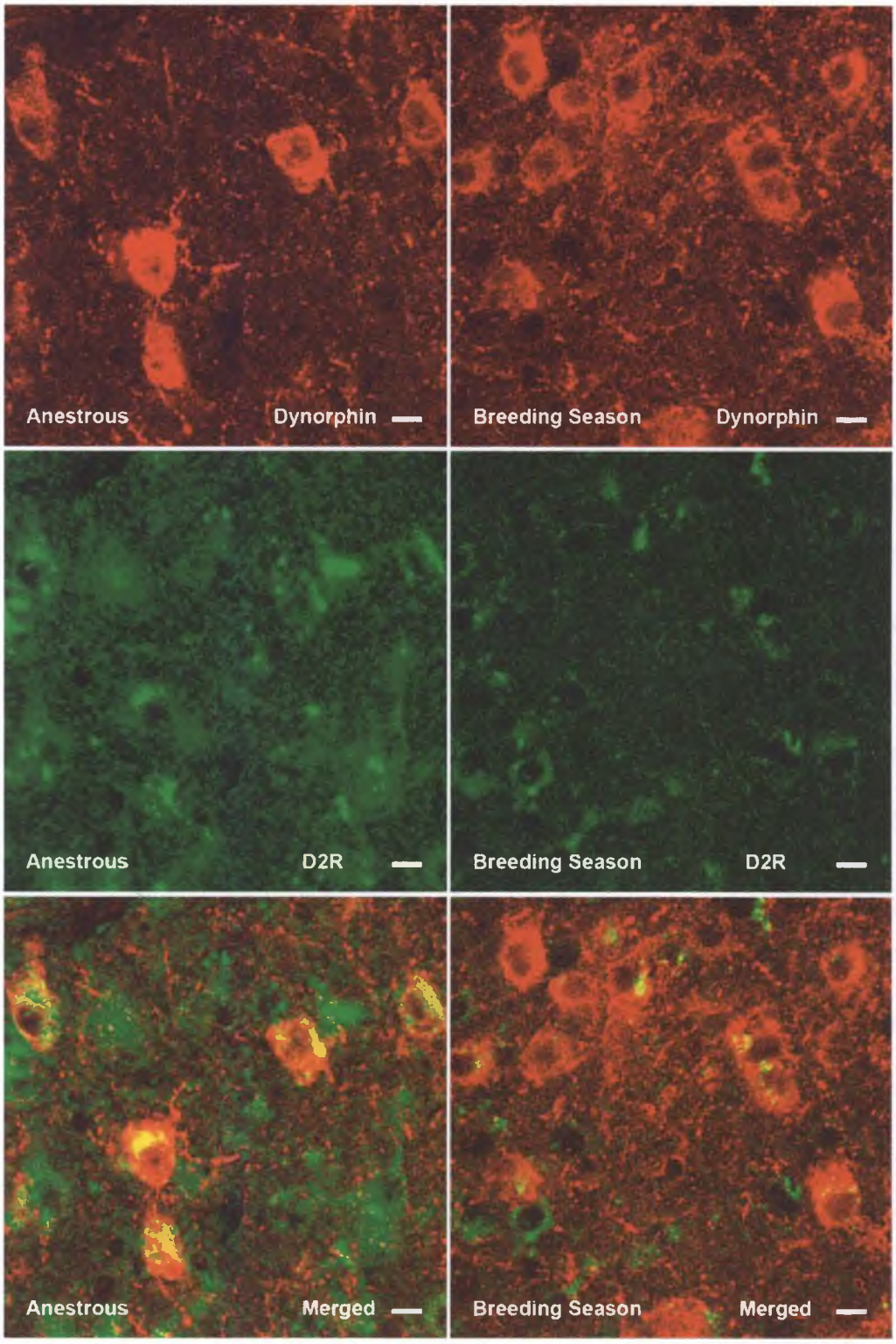


Figure 11 Confocal images of examples of D2R and dynorphin colocalization in the arcuate nucleus of anestrous and breeding season ewes. Dual-label immunofluorescent confocal images (1 µm optical section) of representative dynorphin neurons from the middle arcuate nucleus of ewes in anestrous and breeding season. D2R immunoreactivity is in green, dynorphin in red; colocalization of the signals is shown by yellow. *Scale bar*, 10 µm.

absence of any seasonal differences in the colocalization of D2R in GnRH neurons also argues against this site of action as playing a role in the seasonal ability of dopamine to inhibit GnRH secretion. Nonetheless, it remains possible that A15 dopamine neurons exert their inhibitory influence on GnRH secretion via direct projections onto GnRH cells in the MBH.

In addition to colocalization in GnRH cells, I also found D2R colocalization in KNDy neurons of the arcuate nucleus (ARC), indicating that they too are potential targets of dopamine action. In contrast to our findings in GnRH cells, I found both regional and seasonal differences in D2R colocalization among dynorphin KNDy cells, supporting the possibility that they are intermediaries for conveying the influence of A15 dopamine cells. Specifically, the much higher percentage of D2R colocalization in dynorphin cells in the arcuate nucleus (ARC) than in the preoptic area (POA) is consistent with anatomical evidence that a majority of A15 efferents are directed caudally, including projections to the ARC (Lehman *et al.* 2002). In addition, I found significant seasonal differences in D2R colocalization for ARC dynorphin cells but not for dynorphin cells in either the POA or SON. The direction of this difference is consistent with seasonal changes in responsiveness to E_2 negative feedback, with higher percentages of D2R colocalization seen at the time of year when E_2 negative feedback is maximal. Thus, D2R in KNDy cells are well positioned to convey the inhibitory influence of A15 dopamine in the anestrous brain. Dopamine via D2R may also be involved in regulating the expression of kisspeptin, a neuropeptide known to stimulate GnRH release, as kisspeptin is reduced in KNDy cells during anestrous (Smith *et al.* 2008), when A15 dopaminergic neurons are receptive to E_2 negative feedback (Lehman *et al.* 1996) and D2R expression (this study) is high. Thus, the data taken together suggests the possibility of a bisynaptic pathway from the A15 neurons to GnRH cells via KNDy interneurons, with a potential role in the decreased kisspeptin expression seen in KNDy cells during anestrous. This working hypothesis predicts that KNDy cells should receive direct

synaptic input from A15 neurons; however, whether KNDy cells actually receive inputs dopaminergic synapses, and whether such inputs originate from the A15 cell group, remains to be determined.

As noted above, I observed no obvious regional or seasonal differences in the subcellular localization of D2R in either GnRH or dynorphin cells. Instead, D2R immunoreactive particles appear to be cytoplasmic in both GnRH and dynorphin cells in both seasons, as well as other non-identified neurons observed in the hypothalamus and preoptic area. This may reflect a relative insensitivity of this technique to reflect seasonal differences in the release of endogenous presynaptic dopamine; alternatively, our inability to specifically identify the postsynaptic targets of A15 dopamine cells may not have permitted us to observe any changes in internalization that may exist. To explore the feasibility of this approach further, in the next study, I specifically tested the exogenous D2R agonist application to lead to internalization of the D2R receptor in GnRH and KNDy cells.

2.2 Experiment 2: Does local injection of D2R agonist produce changes in D2R receptor internalization in GnRH and/or KNDy cells?

2.2.1 Rationale

In this experiment, I determined if dopamine acts upon the cells containing D2R by examining ewes who received injections of a local D2R agonist, quinpirole, into the mediobasal hypothalamus in order to drive receptor internalization. Our lab has previously used internalization of G-protein coupled receptors as a marker for endogenous, physiological release of neurotransmitters/neuropeptides in vivo (Coolen *et al.* 2004), and in this study, evidence of receptor internalization following local injections of agonist served as an important positive control. As noted above, there is evidence for dopaminergic terminals directly contacting GnRH

neurons but in very low numbers (Jansen *et al.* 2003); there are no previous reports of whether or not KNDy cells receive direct dopaminergic inputs. Consequently, this experiment will test the ability of GnRH and KNDy cells in the mediobasal hypothalamus to respond to D2R agonist by receptor internalization, and further explore the potential utility of this technique as a marker for analyzing endogenous release of dopamine at a cellular level. Specifically, I hypothesized that injecting a D2R agonist unilaterally into the region of the arcuate nucleus of breeding season ewes would drive the internalization of this G-protein coupled receptor in these cells, leading to the appearance of greater numbers of intracellular particles in the cytoplasm. I examined breeding season ewes, since this is the time of year when endogenous dopamine release is minimal, and therefore, when I might expect to see a maximal effect of D2 agonist upon receptor internalization. In addition, I examined the possibility that exposure to the D2R agonist during the breeding season may increase the percentage of KNDy cells observed as colocalizing D2R.

2.2.2 Methods

Animals and tissue collection

Ovariectomized ewes bearing implants that produced constant, low physiological levels of E_2 were the same as in experiment 1 (see section 2.2.2). As described in Anderson *et al.* (2001), prior to surgery, breeding season ewes (n = 4) were anesthetised with ketamine (7 mg/kg) and diazepam (0.3 mg/kg) and the condition maintained with gas anaesthesia (4:1 N_2O:O_2 plus Halothane [1-5% as needed]). Steel guide cannulae were lowered into small drilled holes in the skull aimed at the middle to caudal arcuate nucleus. Cannulae placement was visualized using a radio-opaque dye (1.5 ml Iohexol) and X-ray radiography. Guide cannulae were cemented in place using dental acrylic and ewes were allowed to recover. Two to four

weeks following cannulation surgery, ewes were given unilateral D2 agonist quinpirole injections through guide tubes and perfused as described above (Experiment 1) approximately 30 minutes after the injection.

Immunocytochemistry

Dual-label immunocytochemical protocols described above were employed on tissue from breeding season ewes with quinpirole injections (n = 4) and a control. Mouse anti-D2R antibody (Santa Cruz Biotechnology) at 1:100 was combined with either mouse anti-GnRH (Sternberger) at 1:500 or rabbit anti-dynorphin A (Phoenix) for dual-label immunofluorescence (see section 2.2.2).

Data analysis

Data was analysed as in experiment 1 (see section 2.2.2). The number of single- and double-labelled GnRH neurons were counted in every section through the mediobasal hypothalamus (MBH) of each animal (control side n = 5, agonist side n = 4) using a Leica DM5000B fluorescence microscope at 20x and 40x magnification (Leica Microsystems). The percentage of GnRH neurons colocalizing D2R in both injected and control sides was calculated for each animal. The mean percentage of GnRH cells colocalizing D2R was then determined for both sides. Similarly the number of single- and double-labelled dynorphin neurons was counted in the arcuate nucleus (4 sections) of each animal (control side n = 5, agonist side n = 4) using a Leica DM5000B fluorescent microscope (Leica Microsystems) with a motorized stage (MBF Biosciences) at 20x magnification. The percentage of dynorphin neurons colocalizing D2R in both injected and control sides was calculated for each animal. The mean percentage of dynorphin cells colocalizing D2R was then determined for both sides. Because of the density of dynorphin

cells in the arcuate nucleus, Neurolucida software (MBF Biosciences) was used as in experiment 1.

MBH GnRH cells colocalizing D2R from both the injected and control sides (n=10 each side) were examined by confocal microscopy. Similarly 10 dynorphin cells in the arcuate nucleus colocalizing D2R from both the injected and control sides were examined. Confocal Z-stacks were acquired at 63x magnification using a LSM 510 META/ConfoCor2 (Carl Zeiss, Inc) with LSM-510 META image processing software. A 543nm emission filter was used on a HeNe laser to view Alexa 555 fluorescent staining. A 488nm emission filter was used on an Argon laser to visualize the Alexa-488 fluorescent staining. Z-stacks were produced with images captured at 1μm intervals through the entire neuron (~20 μm).

Statistical comparisons were made in the number of GnRH and dynorphin cells analyzed in the injected and control side using unpaired student's t-tests. Similarly, unpaired student's t-tests were used to determine if there is a difference in the percentage of D2R expressed in either cell population between the control side and injected side. Differences between means were considered statistically significant if the p value obtained was less than 0.05.

2.2.3 Results

Agonist treatment had no effect on the number of GnRH cells in the arcuate nucleus (Figure 12). Although there was a trend toward a decreased number of immunoreactive dynorphin cells on the agonist-injected side (Figure 12), this difference did not reach significance ($p = 0.07$). Quinpirole had no effect on the percentage of GnRH or dynorphin cells colocalizing D2R (Figure 13). As in Experiment 1, confocal observations revealed no obvious differences between control and injected sides in D2R colocalization within either GnRH or dynorphin

neurons; specifically, in all cells analysed, D2R immunoreactivity appeared as intracellular particles, with a similar number of particles within individual neurons (Figure 14).

2.2.4 Conclusions

The D2R agonist, quinpirole, had no effect on the percentage of cells coexpressing D2R in either GnRH or KNDy cell populations, and percentages of colocalization observed were comparable to that seen in the breeding season animals of Experiment 1 (Figures 6, 9). Similarly, I saw no obvious differences in intracellular localization between control and agonist-injected sides, and, in every case, D2R immunoreactivity within GnRH and KNDy neurons appeared as particles within the cytoplasm. There are several important caveats to this conclusion, however. First, I did not perform quantitative analyses on our confocal material, and thus cannot exclude the possibility that subtle but significant differences exist in the localization of D2R in neurons on injected vs. control sides. A related limitation is that I did not use additional markers in this study to confirm that cytoplasmic D2R immunoreactivity in GnRH or KNDy cells was associated with the endosomal compartment. It is possible that analysis of a more discrete subcellular localization using such markers would reveal agonist-induced internalization. Another potential caveat to the lack of any obvious changes in intracellular localization in this study is that only one dose of agonist was employed, and because injections were only done unilaterally, I was not able to confirm whether this dose is sufficient to inhibit LH pulse frequency during the breeding season. Finally, it is possible that the baseline level of endogenous dopamine release, even in the breeding season, is sufficient to produce the appearance of predominantly intracellular receptors. Thus, a more feasible approach in the future may be to apply an antagonist in order to block internalization in either anestrous or breeding season animals.

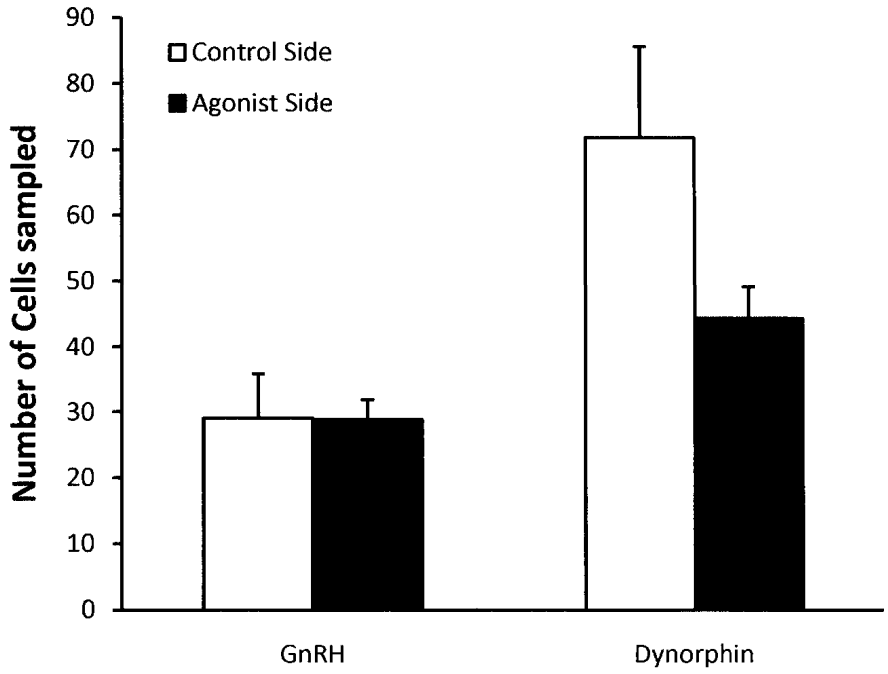


Figure 12 Total number of GnRH and dynorphin cells (mean ± SEM) analysed in MBH of breeding season ewes that received unilateral injections of D2R agonist. No significant differences between control and injected sides of the brain were found using unpaired student's t-tests in number of GnRH or dynorphin cells analysed (control side n = 5, agonist side n = 4).

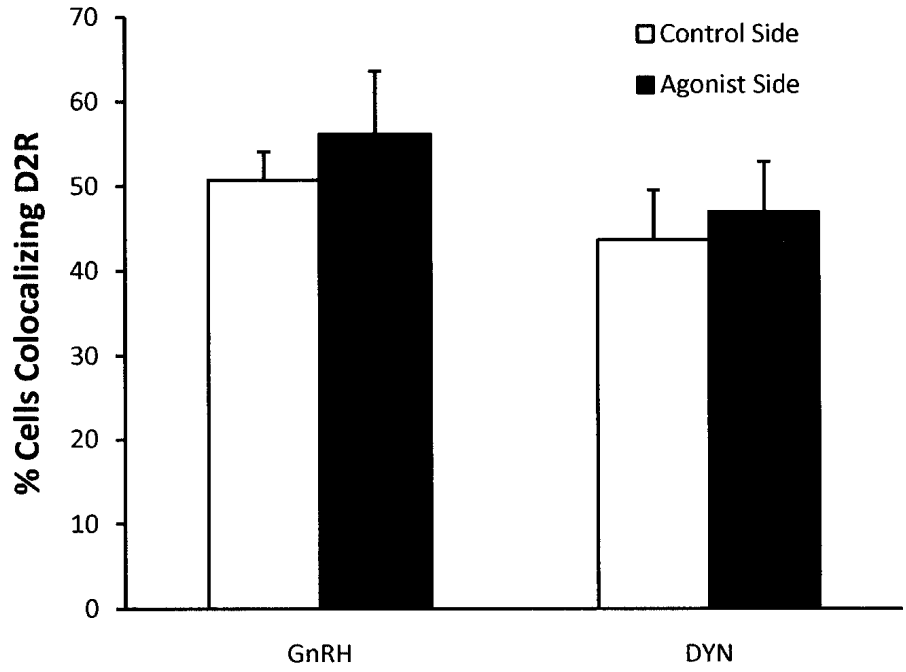


Figure 13 Percentage colocalization of D2R and either GnRH or dynorphin cells (mean ± SEM) in the MBH of breeding season ewes that received unilateral injection of D2R agonist into the arcuate nucleus. No significant effect of the agonist on percentage of colocalization of D2R were found using unpaired student's t-tests in GnRH cells or dynorphin cells (control side n = 5, agonist side n = 4).

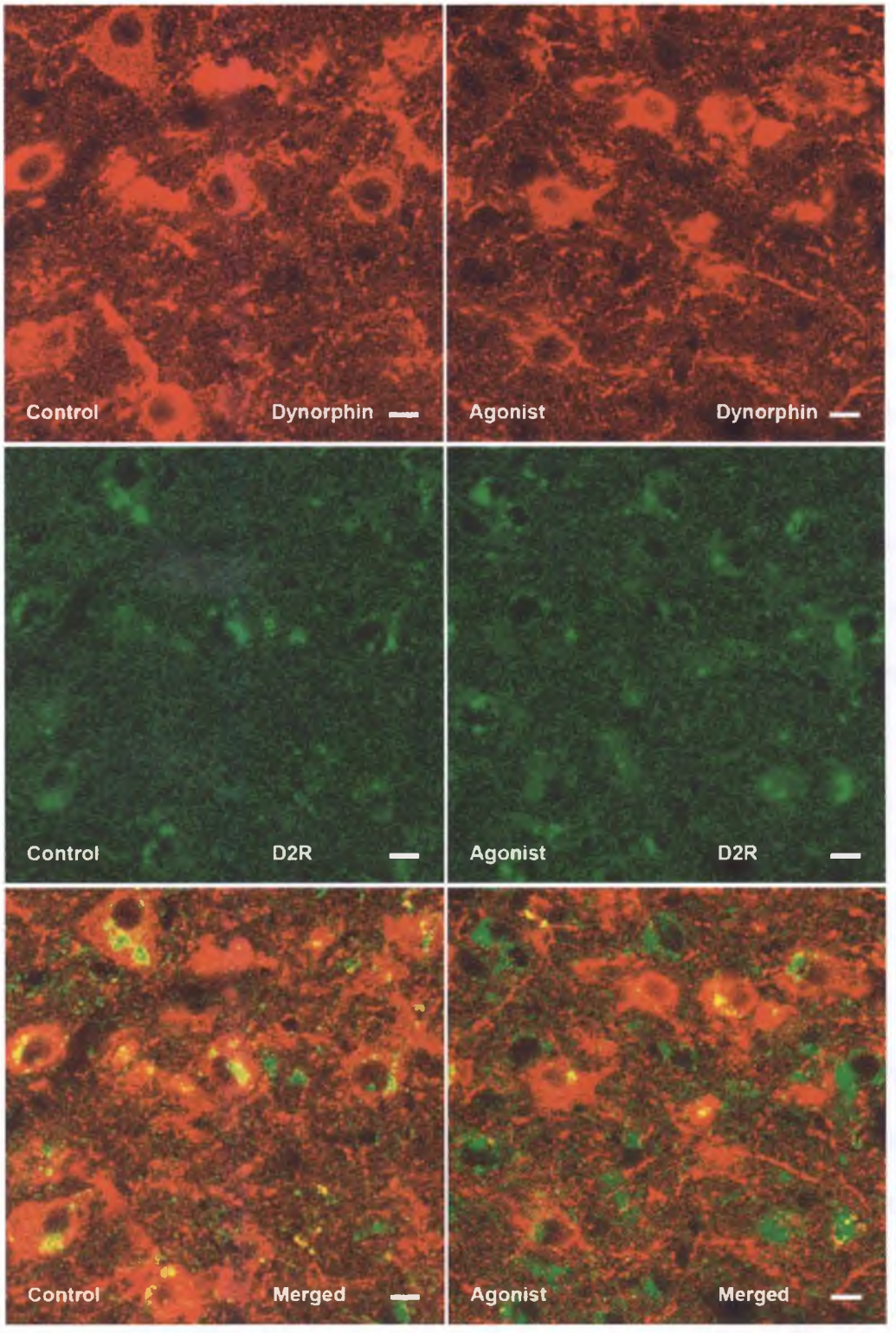


Figure 14 Confocal images of examples of D2R and dynorphin colocalization in control and D2 antagonist-injected sides of the brain. Dual-label immunofluorescent confocal images (1 µm optical section) of representative dynorphin neurons from the middle arcuate nucleus of ewes from the control side and agonist injected side. D2R immunoreactivity is in green, dynorphin in red; colocalization is indicated by yellow. *Scale bar*, 10 µm.

2.3 Experiment 3: Do KNDy cells project directly to GnRH neurons

2.3.1 Rationale

If KNDy cells serve as a critical intermediary in a bisynaptic pathway conveying the influence of dopamine onto GnRH neurons, then a strong prediction of this hypothesis is that KNDy terminals should provide direct synaptic input to GnRH cells. Although close contacts between kisspeptin fibres and terminals and GnRH somas and dendrites have been described in rodents (Clarkson and Herbison 2006) and monkeys (Ramaswamy *et al.* 2008), they have not been systematically examined in sheep, and confirming their synaptic nature would provide further evidence for the role of KNDy cells in conveying the inhibitory influence of E_2 via A15 dopaminergic neurons. Therefore, I used triple-label immunocytochemistry and confocal microscopy to determine if kisspeptin contacts onto GnRH cells are bona fide synapses, using the synaptic vesicle protein, synaptophysin, as a marker.

2.3.2 Methods

Animals and tissue collection

Animals were maintained and tissue collected described in Smith *et al.* (2008). Briefly, adult Suffolk black-faced ewes were housed in ambient environment with a maintenance ration of food and free access to water at the West Virginia University Research Farm. Ewes were ovariectomized and given a subcutaneous E_2 implants to maintain constant, low levels of circulating E_2. Ewes in anestrous and the breeding season were sacrificed with an overdose of sodium pentobarbital, the heads perfused bilaterally with 6 litres of 4% PFA in 0.1M PB, and dissected hypothalamic blocks stored overnight in 4% PFA in 0.1M PB. Tissue was stored at -20°C in cryoprotectant solution (0.004% Sodium Azide, 30% ethane-1, 2-diol, and 300g sucrose per L

in 0.1M PB) until coronal sections of 50μm were cut on a freezing microtome. Tissue sections were stored at -20°C in cryoprotectant.

Immunocytochemistry

The immunocytochemical protocol employed was similar to that described above (see 2.2.2) but with three different primary antibodies: GnRH, kisspeptin, and synaptophysin. The first primary antibody used was rabbit anti-kisspeptin (A. Caraty, Université de Tours) at 1:300000 in PBS+, amplified as before using the using the tyramide signal amplification system, and visualized using as Cy5 conjugated streptavidin (Jackson laboratories) at 1:100 in PBS. The second primary antibody, rabbit anti-GnRH (LR5), was incubated at 1:1000 in PBS+ while simultaneously incubating with the third primary antibody, mouse anti-synaptophysin (Sigma) at 1:1000 in PBS+. They were respectively visualized by incubation in the secondary antibodies Alexa Fluor 555 goat anti-rabbit (Molecular Probes) 1:100 in PBS followed by incubation in Alexa Fluor 488 goat anti-mouse (Molecular Probes) 1:100 in PBS. All primary antibodies incubated for 17 hours, and all fluorophore-conjugated secondary antibodies for 30 minutes. Immunocytochemical controls included omission of each primary antibody by itself which resulted in the elimination of immunofluorescent signal specific to that antigen, and did not affect staining for the other two antigens. In addition, previous studies have performed antigen preabsorption controls in sheep tissues for each of the three primary antibodies used and showed them to completely eliminate all staining for the corresponding antibody (kisspeptin: Goodman *et al.* 2007; GnRH: Goodman *et al.* 2004; synaptophysin: Adams *et al.* 2006). The kisspeptin antibody used is specific for kisspeptin-10, and does not recognize any other related RFamide peptide (e.g., GnIH; Goodman *et al.* 2007).

Data analysis

Previous counts at the light microscope level found significantly greater kisspeptin contacts onto GnRH neurons in the AHA and MBH in breeding season compared to anestrous (Smith *et al.* 2008). The synaptic nature of these contacts were confirmed using a confocal Z-stacks (captured as before, see 2.2.2) of 5 cells from the MBH in anestrous (n=4) and breeding season (n=5) ewes. An additional sample of 5 cells from the POA of anestrous and breeding season ewes was also captured. Images were analysed using Leica Image Viewer (Leica Microsystems), positive close contacts were defined as a group of pixels approximately 1μm in diameter that were pseudo-coloured magenta, indicating immunoreactivity of both kisspeptin (red) and synaptophysin (blue), directly bordering pixels of green GnRH immunoreactivity in either the soma or dendrite of the cell.

2.3.3 Results

Previous data has shown around half of GnRH cells in the POA, regardless of season, half of GnRH cells in the MBH of anestrous ewes, and nearly all GnRH cells in the MBH of breeding season ewes have kisspeptin contacts (Smith *et al.* 2008). Of those neurons with contacts, I analysed around 25 cells per region sampled from ewes in anestrous or breeding season. In all cases where I observed kisspeptin terminals in close contact with GnRH somas or dendrites in the POA or MBH, in each and every instance, those same contacts were immunopositive for synaptophysin, confirming their synaptic nature (Figure 15).

2.3.4 Conclusions

The confirmation that kisspeptin contacts onto ovine GnRH neurons observed with confocal microscopy in 1 μm thick optical sections also contain synaptophysin provides strong

evidence that these contacts represent bona fide synapses. In addition, previous studies have shown, using another synaptic vesicle marker (synapsin), that when such close contacts are observed at a light level, when the same material is analysed at an electron microscopic level, every one of those light microscopic associations are shown to be synaptic in nature (Adams *et al.* 2006). Thus, I have confidence that the observations reported here represent a valid reflection of what one would expect to see at an ultrastructural level where synapses can be identified on the basis of organelles (e.g., vesicles) and synaptic specializations.

Detection of kisspeptin synaptic terminals onto GnRH soma and dendrites supports the role of the KNDy cell population as a key component of the E_2 negative feedback pathway. Such a role is consistent with seasonal comparisons showing a decrease in kisspeptin mRNA and peptide in the arcuate nucleus of anestrous ewes compared to breeding season ewes (Smith *et al.* 2008). Thus, one of the actions of dopamine on KNDy cells may be to down-regulate kisspeptin expression. Since kisspeptin is an excitatory neuropeptide and is extremely stimulatory to GnRH secretion (e.g., at femtomolar concentrations), I hypothesize that a downregulation of kisspeptin expression and release in anestrous allows for inhibitory peptides expressed by KNDy cells (e.g., dynorphin) to predominate and functionally inhibit GnRH secretion at that time of year. Consistent with this working hypothesis are observations that the number of kisspeptin contacts onto GnRH cells, specifically those in the MBH, is reduced in anestrous ewes, further supporting the role of this cell population as a mediator in the E_2 negative feedback pathway (Smith *et al.* 2008). Thus, the data provided by these experiments suggest that the kisspeptin and GnRH components of this pathway are both located in the MBH: dopaminergic fibres from the A15 contacting D2R-containing kisspeptin (KNDy) cells in the arcuate nucleus, and KNDy cells, in turn, provide input to neighbouring GnRH neurons, ultimately inhibiting GnRH and LH pulse frequency in anestrous (Figure 16).

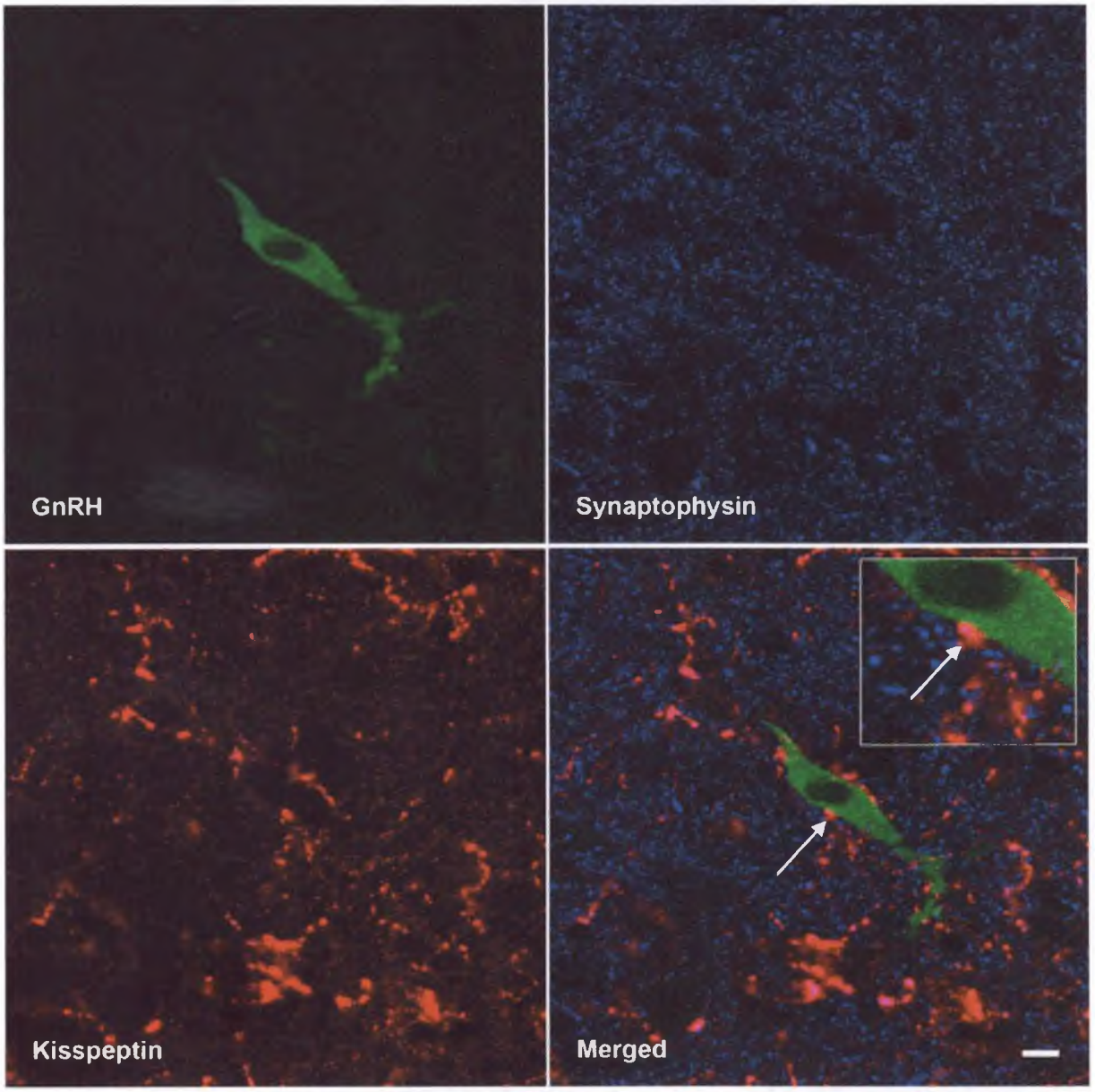


Figure 15 Example of synaptophysin-positive kisspeptin contacts onto a GnRH cell. Triple-label immunofluorescent confocal images (1 µm optical section) of kisspeptin close contacts onto a GnRH cell in the mediobasal hypothalamus. Arrow indicates an example of a synaptophysin-positive kisspeptin close contact. *Scale bar*, 10 µm.

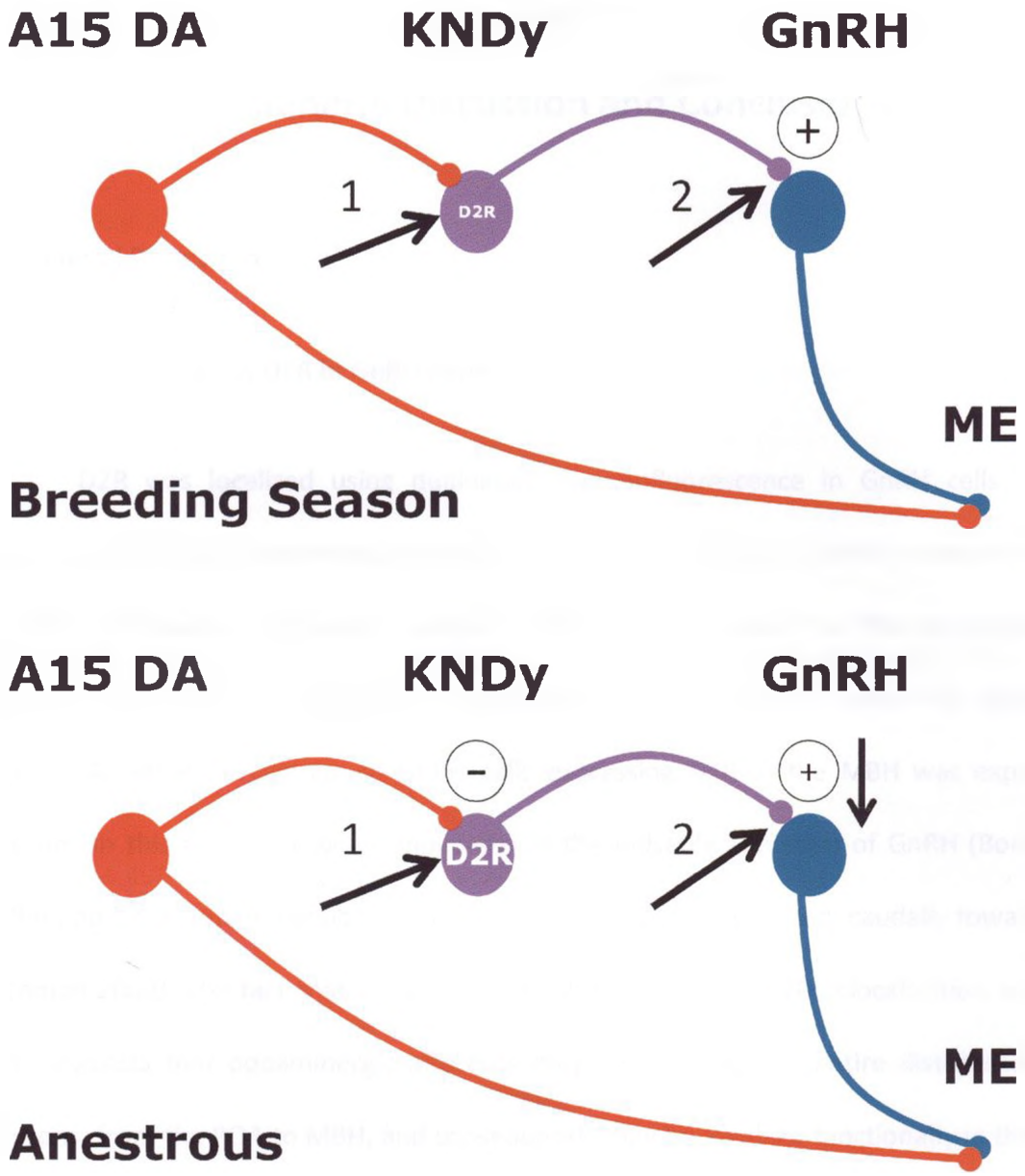


Figure 16 Components of the hypothesized E_2 negative feedback pathway added by this research. Two components were added to the current working model of E_2 negative feedback shown in Fig. 1: (1) the presence of dopamine D2 receptors in KNDy cells of the arcuate nucleus; and (2) kisspeptin-positive synaptic inputs onto GnRH cells. Thus, we propose that E_2 negative feedback in conveyed by A15 dopamine neurons to D2R-containing KNDy cells in the arcuate nucleus, and that inhibition of stimulatory KNDy cells, in turn, is responsible for inhibition of GnRH and LH pulse frequency in anestrous. D2R: dopamine D2 receptor; DA: dopamine; GnRH: Gonadotropin-releasing hormone; KNDy: Kisspeptin/neurokinin B/dynorphin neurons of the arcuate nucleus; ME: median eminence.

Chapter 3

General Discussion and Conclusions

3.1 General Discussion

3.1.1 Colocalization of D2R in GnRH neurons

D2R was localized using dual-label immunofluorescence in GnRH cells to test the hypothesis that A15 dopaminergic neurons directly inhibit pulsatile GnRH secretion at the level of GnRH cell bodies. D2R were found in GnRH cells throughout the POA and hypothalamus; however the percentage of GnRH neurons expressing D2R did not vary with region or with season. A higher percentage of GnRH cells expressing D2R in the MBH was expected since neurons in this area have been implicated in the pulsatile secretion of GnRH (Boukhliq *et al.* 1999) and since dopaminergic neurons from the A15 project primarily caudally toward the MBH (Lehman 2002). The fact that I saw no regional differences in D2R colocalization among GnRH cells suggests that dopaminergic afferents may be affecting the entire distribution of GnRH neurons, from the POA to MBH, and consequently may be involved functionally in the control of surge as well as pulsatile modes of GnRH secretion.

Similarly, a lower percentage of GnRH cells from breeding season ewes were predicted to express D2R since GnRH neurons at this time are dramatically less responsive to the negative feedback of E_2. A lack of seasonal variation weakens the hypothesis that A15 neurons act directly on GnRH cell bodies to inhibit GnRH secretion in anestrous. However, it may be that seasonal plasticity is exerted primarily at the level of A15 dopamine neurons (Adams *et al.* 2006), and is not necessarily reflected by changes at the level of postsynaptic dopamine receptors. It is also possible that another source of dopaminergic fibres other than the A15 cell group is acting on

D2R in GnRH cells; one such possibility would be dopaminergic cells in the A12 group which sparsely project to the preoptic area in sheep (Dufourny *et al.* 2005). Regardless, our results indicate that it is unlikely that the seasonal variation in this system can be accounted for by D2R expression in GnRH cells alone.

It is possible that I am detecting D2R being created in the cell body and stored prior to being transported to axon terminals, including those in the median eminence. Previous studies have shown TH positive terminals in direct contacts with GnRH terminals in the median eminence suggesting an axo-axonic mode of communication (Kuljis and Advis, 1989) and local administration of D2R agonist and antagonist at the level of the median eminence have been shown to modulate LH secretion (Bertrand *et al.* 1999). However, using the antibody employed in this study I did not observe any D2R immunoreactivity in the median eminence, nor did I see colocalization of D2R in GnRH axons in any other region. It may be that levels of D2R found at the terminal may be too low to be detected by immunofluorescence despite the use of the tyramide signal amplification system. Alternatively, effects of D2R agonists and antagonists delivered at the level of the median eminence upon LH pulses (Bertrand *et al.* 1999) may be due to diffusion to the nearby arcuate nucleus, the site of KNDy cells.

3.1.2 Colocalization of D2R in KNDy neurons

Dual-label immunofluorescence was used to colocalize D2R and dynorphin, the latter used as a marker for KNDy cells, in the arcuate nucleus to test the hypothesis that A15 dopaminergic neurons inhibit GnRH secretion indirectly via KNDy interneurons. Colocalization of D2R and dynorphin was seen in a majority of the KNDy cell population but only very sparsely among dynorphin cells in the POA. This finding is consistent with studies implicating the KNDy population in the sex steroid negative and positive feedback regulation of GnRH (Smith *et al.*

2007). As A15 neurons have been shown to project to the MBH (Lehman *et al.* 2002), the location of KNDy cells in this region supports the hypothesis that they act as an interneuron between A15 and GnRH neurons. An important caveat of this conclusion is that I have not yet demonstrated that KNDy cells receive direct dopaminergic input, nor do I know that such input arises from the A15 since tract tracing from the A15 was not combined with this study. Nevertheless, the KNDy cell population remains a very strong candidate for conveying the inhibitory influence of dopamine to GnRH in anestrous.

Seasonal differences were also found in D2R coexpression in KNDy cells. A marked decrease in the percent of KNDy cells expressing D2R was found in breeding season. This is again consistent with the hypothesized model that KNDy cells are interneurons in the E_2 negative feedback pathway (Fig. 13). Specifically, these findings suggest the intriguing possibility that dopamine from the A15 may act via D2R in KNDy cells to inhibit expression of the stimulatory peptide kisspeptin during anestrous. This provides a possible explanation for the observed decrease in kisspeptin mRNA and peptide levels in the KNDy subpopulation, as well as the decrease in the number of kisspeptin contacts onto GnRH neurons in anestrous (Smith *et al.* 2008). Furthermore, ovariectomy increases KNDy kisspeptin mRNA and peptide in anestrous ewes, supporting a steroid dependent mechanism of gene regulation (Smith *et al.* 2007). It is important to note that KNDy cells express multiple neuropeptides: the excitatory neuropeptides, kisspeptin and neurokinin B, and the inhibitory peptide, dynorphin. All three peptides are contained in terminals which are presynaptic to GnRH neurons, and it has been hypothesized that the balance of neuropeptides expression and release is a major factor in the steroidal regulation of GnRH secretion (Cheng *et al.* 2009). Thus, dopamine from the A15, in concert with D2R, may act as a possible neuroendocrine switch altering the balance of excitatory vs.

inhibitory neuropeptide release from KNDy cells onto GnRH cells, thereby accounting for the dramatic seasonal shift in sensitivity of GnRH cells to E_2 negative feedback.

3.1.3 Effect of D2R Agonist quinpirole on D2R expression

The agonist quinpirole was injected unilaterally into the mediobasal hypothalamus of ewes in the breeding season in order to drive internalization of D2R, as a marker of activation. This would confirm that dopamine acts upon D2R as there have been only very few TH contacts found on GnRH cells and no reports of TH contacts onto KNDy cells. Unfortunately, nearly all D2R immunoreactivity appeared to be cytoplasmic in GnRH and KNDy neurons on both control and agonist-injected sides of the brain. It was consequently not possible to detect differences in internalization between treatment and control sides of the hypothalamus in either cell population. It may be that sufficient levels of endogenous dopamine release occur to internalize available D2R regardless of season, with so few D2R remaining membrane-bound that I could not detect them with the immunocytochemical approach used. However, since I did not employ double-labelling for endosomal markers I could not definitely conclude that the D2R immunoreactivity I observed represented receptors internalized following ligand binding. For example, some of the cytoplasmic D2R immunoreactivity could have been D2R in the endoplasmic reticulum or the Golgi apparatus as part of its trafficking to the membrane or axon terminal. Therefore, a more detailed analysis of the subcellular localization of D2R immunoreactivity in these cells would be worthwhile.

3.1.4 Kisspeptin close contacts onto GnRH cells

I used triple-label immunofluorescence to confirm that kisspeptin contacts onto GnRH cells are synaptic in nature, further strengthening the hypothesized role of KNDy cells as

interneurons in the E_2 negative feedback pathway. The number of contacts onto GnRH cells has been shown to decrease in breeding season, further implicating these cells in the neural control of seasonal GnRH secretion (Smith *et al.* 2008).

3.1.5 Implications in other species

The hypothesized E_2 negative feedback pathway for the ewe (Figure 2) contains elements that are both similar and different to models developed in other species. The presence of E_2 negative feedback has been shown in many species, with pulsatile GnRH secretion being increased in gonadectomized mice, sheep, primates, and humans, and inhibited following E_2 administration (for review see Herbison 1998). As ERα is not colocalized in GnRH cells in any of these species, the inhibitory influence of E_2 in the control of GnRH secretion appears to involve interneurons upstream of the GnRH neuron itself (Herbison 1998). Due to the different reproductive strategies employed by mammals, the pathway transferring the negative feedback action of E_2 onto GnRH neurons is likely to be variable. Specifically, data obtained in other species suggest that GABAerigc neurons from the POA, which are hypothesized in the sheep to convey the inhibitory influence of E_2 in anestrous via dopaminergic A15 neurons to GnRH, may bypass dopaminergic interneurons inhibiting GnRH neurons directly. Evidence for this include: the lack of a well defined A15 cell group in other mammalian species as well as studies demonstrating that ovariectomized rats and monkeys display increased LH secretion and reduced GABA levels in the POA where ERα receptive cells have been located (Herbison 1998). It is thus possible that the A15 region may be an additional component to the E_2 negative feedback pathway in the sheep responsible for seasonal control of reproduction and that this component is not seen in non-seasonal breeders. Supporting this is evidence in the Syrian hamster, another seasonally breeding mammal, in which dopamine content of the mediobasal

hypothalamus/median eminence increases during reproductive quiescence (Benson 1987, Krajnak *et al.* 1995). It is also possible that the dopaminergic aspect of the feedback loop has not yet been discovered in other mammals. Dopamine has, however, been implicated in the control of GnRH in other, more evolutionarily distant species. For example, in the fish *Mugil cephalus,* dopamine has been shown to act via D2R to inhibit secretion of GnRH, although the nature of this role is unknown (Aizen *et al.* 2005) and unlikely to be related to mammalian E_2 negative feedback system. Further comparative studies are needed to confirm the role of dopamine and D2R in the E_2 negative feedback pathway in other species. Although evidence for seasonality in human reproduction is controversial (Bronson 2004), understanding how this system evolved and functions will ultimately improve our understanding of how disruptions in E_2 negative feedback may lead to the reproductive neuroendocrine diseases in humans.

3.2 Future Directions

Although the neural pathways mediating seasonal control of GnRH secretion has been a topic of active investigation for many years, numerous aspects of this circuitry remain unknown. Specifically, there was a paucity of information connecting dopamine from the A15 to the inhibition of GnRH secretion via D2R. Our study has provided key pieces of information filling this gap; however much remains to be discovered.

Dopaminergic contacts have been shown to sparsely innervate GnRH cells but no study has as yet examined dopaminergic inputs onto cells of the KNDy subpopulation. Triple-label immunofluorescence should be employed to identify tyrosine hydroxylase (TH)-positive contacts onto KNDy cells that express D2R. Tract tracing will be needed to confirm that the source of these dopaminergic afferents is from the A15 region. Combined with the finding that KNDy cells

project to GnRH, this data would be necessary to confirm the bisynaptic nature of this portion of the E_2 negative feedback system.

The possible presynaptic role that D2R may play in conveying the dopamine signal from the A15 is also worth exploring. Confocal images taken in this study also show D2R immunoreactivity within terminals that themselves are in close contact with GnRH and KNDy cells. This suggests that presynaptic short-form D2R autoreceptors might exist in dopaminergic terminals on GnRH and KNDy cells regulating dopamine release from the A15. Triple-label fluorescence staining for dynorphin, D2R, and synaptophysin, a marker for synapses, combined with confocal analysis would confirm that these are indeed close contacts. Seasonal variation expression of D2R in these contacts could provide another element of the feedback loop that may be potentially responsible for the dramatic increase in sensitivity of GnRH neurons to the negative feedback of E_2 in anestrous.

Nearly all D2R immunoreactivity appears to cytoplasmic; however the intracellular position of these receptors remains unknown. Triple-label immunofluorescence with dynorphin, D2R and intracellular markers combined with confocal microscopy will help understand the role of D2R in this system. Specifically, if antibodies for internalized vesicle proteins (e.g., endosomal proteins) can be combined with D2R and dynorphin, this would strengthen the hypothesis that the receptors being detected are those that are internalized as a result of endogenous ligand binding. Markers for the Golgi apparatus or endoplasmic reticulum can similarly be combined to indicate that the receptors are being stored after translation, and prior to be transported to somatic or dendritic membranes, or down the axon. Alternatively, activation can be inferred with a unilateral injection of a D2R antagonist into the MBH of anestrous ewes to see if it blocks internalization of D2R in either GnRH or KNDy neurons.

It will also be important to test the possibility that D2R is involved in the seasonal switch in the ability of E_2 to inhibit GnRH secretion. As D2R expression is increased in anestrous when the stimulatory peptide kisspeptin is decreased, it is hypothesized that dopamine acting via D2R may be responsible. Further studies are needed to confirm the potential role of dopamine and D2R in regulating gene/peptide expression in the KNDy subpopulation. For example, long-term administration of a D2R antagonist into the MBH of anestrous ewes may reverse the seasonal decrease seen in kisspeptin mRNA and peptide expression seen in the arcuate nucleus of anestrous ewes (Smith *et al.* 2008) and increase kisspeptin expression to levels normally only seen in breeding season ewes.

Other cell populations in the MBH expressing D2R might also be interneurons modulating the influence of dopamine from the A15 on GnRH secretion. Our study found many cells that express D2R in the arcuate nucleus that do not belong to the KNDy cell population, and I saw what appeared to be an increase in D2R immunoreactivity in many non-KNDy cells in the arcuate nucleus of anestrous ewes (Figure 11). Other neuronal subpopulations of the arcuate nucleus have been implicated in neuroendocrine control: these include agouti-related protein, pro-opiomelanocortin, and orphanin FQ expressing cell populations (Xu *et al.* 2009, Foradori *et al.* 2007). In addition, dopamine cells of the A12 cell group, the tuberoinfundibular dopamine system, are in close proximity to the KNDy cells studied here. A12 dopamine cells play a major role in the regulation of prolactin secretion, although seasonal control of prolactin in the sheep is primarily exerted at a pituitary level (Lincoln and Clarke 2002). Nonetheless, there is evidence for interactions between A12 dopamine cells and GnRH cell bodies and terminals in the median eminence in the sheep (Lehman *et al.* 1987), as well as A12 projections to the vicinity of GnRH cells in the preoptic area (Dufourny *et al.* 2005).

Finally, despite the fact that humans are not seasonal breeders, it is likely that a basic understanding of the brain pathways by which E_2 inhibits GnRH secretion in the ewe will have relevance for the treatment of human disease. As noted in the introduction, disruptions in E_2 negative feedback control of GnRH underlie many reproductive neuroendocrine pathologies in humans. For example, increased responses to E_2 negative feedback is seen in women anorexia nervosa (Couzinet *et al.* 1999) and hypothalamic amenorrhea (Quigley *et al.* 1980), and there is evidence for increased DA inhibition of LH in the latter in that patients given the dopamine D2 receptor antagonist, metoclopramide, show elevated LH and FSH secretion (Quigley *et al.* 1980). By contrast, an apparent decreased response to E_2 negative feedback occurs in polycystic ovary syndrome (Morales *et al.* 1996). The neuroendocrine basis for the disruption of E_2 negative feedback in PCOS patients is unclear, but dopamine may be involved since metoclopramide fails to alter LH secretion in women with PCOS, an effect seen in normal cycling women (Cumming *et al.* 1984). In addition, much recent attention has been focused on the potential that kisspeptin and NKB agonists and antagonists may have in the treatment of this disorder and other reproductive dysfunction (Roseweir *et al.* 2009). Thus, a more complete understanding of the role of dopamine cells, D2R and KNDy peptides in the steroid feedback control of reproductive neuroendocrine events, may have far-reaching consequences in the attempt to develop better treatments for human reproductive disorders involving E_2 negative feedback mediated ovarian dysfunction.

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