The Effect of GLP-1 on the Reproductive Axis of the Ewe

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Certificate of Authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the dissertation. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged. I agree that this dissertation be accessible for the purpose of study and research in accordance with normal conditions established by the Executive Director, Library Services, Charles Sturt University or nominee, for the care, loan and reproduction of dissertation, subject to confidentiality provisions as approved by the University.

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Chapter 4 has been published in a peer-reviewed journal as outlined below. Elizabeth Simpson was responsible for composing the manuscript, however the collaborating authors contributed to the revision of the article and approved the final manuscript for publication.

I, Allan Gunn (senior author), sign on behalf of the collaborating authors to confirm the above statement and consent to the inclusion of the publication in this dissertation.

Allan Gunn

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Ethics Statement

Animal care and ethics approval was attained prior to sample collection. The Animal Care and Ethics Committee, Charles Sturt University approved all study protocols. The approved protocol numbers are A19385, A19005 and A18029.

Publications and Conferences

Simpson, EM, Clarke, IJ, & Scott, CJ, Stephen, CP, Rao, A and Gunn, AJ (2023) The GLP-1 agonist, Exendin-4, stimulates LH secretion in female sheep. The Journal of endocrinology. 10.1530/JOE-23-0105.

Jones, EM (2018), The missing link? GLP-1 and reproduction, *School of Animal and Veterinary Sciences Research Day,* Charles Sturt University, Wagga Wagga NSW, oral presentation

Jones, EM (2019) A missing link? Nutrition, reproduction and the role of GLP-1, *Higher Degree by Research and Honours Symposium,* Charles Sturt University, Wagga Wagga NSW, oral presentation

Abstract

The relationship which exists between nutrition and reproduction is well established but poorly understood. Recent studies suggest that the gut peptide, GLP-1, may have a stimulatory effect on the hypothalamic-pituitary-gonadal axis, increasing secretion of GnRH and/or pituitary gonadotrophs (Arbabi, Li, Henry, & Clarke, 2021; Farkas et al., 2016; Outeirino-Iglesias, Romani-Perez, Gonzalez-Matias, Vigo, & Mallo, 2015; C. Vastagh, Farkas, Scott, & Liposits, 2021). However, whether GLP-1 in systemic circulation can increase gonadotroph secretion is unknown, with contradictory results from a study in rats which found that although GLP-1 seemed to stimulate the reproductive axis, administration of the GLP-1 receptor agonist, Exendin-4, had some inhibitory effects (Outeirino-Iglesias et al., 2015)

A preliminary study confirmed proof-of-concept, with intravenous infusion of 2mg of Exendin-4 via indwelling jugular catheter over a one-hour period increasing the secretion of luteinising hormone (LH) in a population of ewes. Oestrus synchronization was used to allow observation of whether this effect differed between the luteal or follicular phase of the oestrous cycle. The effect of Exendin-4 infusion on LH secretion was markedly more pronounced in the follicular phase.

A larger study was then designed using solely ewes in the follicular phase and confirmed the effect of Exendin-4 infusion on LH secretion at a dose of 0.5mg or 2mg. Although Exendin-4 infusion increased LH pulse amplitude, there was no observable effect on inter-pulse interval. Measurement of plasma insulin and glucose concentrations confirmed these dosages were not associated with an incretin effect. There was no observed effect of Exendin-4 infusion on timing of the preovulatory LH surge.

Data from the preliminary study suggested that Exendin-4 also stimulated follicle stimulating hormone (FSH) secretion, however the results of the main experiment suggest we observed an endogenous increase and that Exendin-4 infusion does not have an immediate effect on FSH secretion.

Glucagon-like peptide 1 is produced by post-translational processing of the preproglucagon gene. Preproglucagon gene (*GCG*) expression has not previously been reported in the sheep. We confirmed that *GCG* is expressed in the ovine intestine using tissue samples collected from seven sites of the gastrointestinal tract of five ewes. Quantitative PCR was utilized to assess differences in gene expression between sites. Expression increased aborally, being greatest in the rectum and lowest in

the forestomachs. This distribution similar to *GCG* expression in cattle. This suggests the distribution of intestinal L-cells in ruminants is similar to that of monogastric species.

We can conclude that GLP-1 in systemic circulation, such as that produced by the hind-gut, acts to increase LH secretion in the ewe. These findings are consistent with the hypothesis that GLP-1 has a stimulatory effect on the hypothalamic-pituitary-gonadal axis.

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Glossary of abbreviations.

AgRP	Agouti-related peptide
ARC	Arcuate nucleus
CART	Cocaine-and-amphetamine-regulated transcript
CCK	Cholecystokinin
CL	Corpus luteum
CRH	Corticotropin-releasing hormone
DMI	Dry matter intake
DMN	Dorsomedial nucleus
DPP IV	Dipeptidyl peptidase IV
E2	Oestradiol 17β
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GCG	Preproglucagon gene
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide 1
GLP1R	Glucagon-like peptide 1 receptor
GLP-2	Glucagon-like peptide 2
GnIH/RFRP3	Gonadotrophin-inhibitory hormone
GnRH	Gonadotrophin-releasing hormone
HPG axis	Hypothalamic-pituitary-gonadal axis
KNDy	Kisspeptin, neurokinin B and dynorphin expressing neurons
LH	Luteinising hormone
LHA	Lateral hypothalamic area
ME	Median eminence

mRNA	Messenger RNA
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OXM	Oxyntomodulin
P4	Progesterone
PCOS	Poly-cystic ovarian syndrome
PGF2α	Prostaglandin F2α
POA	Pre-optic area
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY
qPCR	Quantitative PCR/Real-time PCR
SCFA	Short-chain fatty acid
T2DM	Type 2 diabetes mellitus
TH	Thyroid hormone
TRH	Thyrotropin-releasing hormone
VFA	Volatile fatty acid
VMN	Ventromedial nucleus

Chapter 1: Introduction

Chapter 1: Introduction

The regulation of energy and reproduction is central to the two core drives of any higher order animal; 1) to survive and 2) to reproduce. Philosophers as early as Aristotle observed that nutrient supply to an animal can have a profound impact on reproductive success, and it is likely this understanding has been appreciated for considerably longer (Comninos, Jayasena & Dhillo, 2014; Martin, Blache, Miller, & Vercoe, 2010). Animals in a “negative” energy balance display a multitude of possible issues, including delayed puberty, reduced ovulation and conception rates, irregular or absent cyclicity, poorer quality gametes, poorer quality embryos etc. (Chelikani, Ambrose, & Kennelly, 2003). We now know that some effects of an energy-deficit can be multi-generational as a consequence of epigenetic programming *in utero* (Sharpe, 2018). Conversely, “over-nutrition”, such as obesity, is also associated with reduced fertility (Comninos, Jayasena, & Dhillo, 2014; Robinson, Ashworth, Rooke, Mitchell, & McEvoy, 2006).

It is surprising, therefore, that relatively little is known about how these two critical axes, metabolism and reproduction, communicate. It is beyond doubt that metabolism can influence reproduction and can do so in an indirect manner, due to observations such as the upregulation of luteinizing hormone (LH) secretion that occurs before any changes in body condition or substrate availability that occurs when fasted animals are re-fed (De Bond & Smith, 2014; Henry et al., 2004). The physiology that underlies regulation of reproduction by metabolism (or a reciprocal relationship), is largely unknown. Research into the effects of the adipokine, leptin, on the reproductive axis, have confirmed that

although it has an important role in the signaling of adequate energy reserves, it is only one part of the picture (Henry et al., 2004). The question remains: how does the hypothlalamic-pituitary-gonadal axis receive information that an animal is in a state of positive or negative energy balance and respond accordingly?

Recent candidates for a role in this relationship are the gut peptides, including glucagon-like peptide 1 (GLP-1). GLP-1 is primarily produced in the intestine, with some central secretion, by post-translational processing of the preproglucagon gene. The preproglucagon gene is expressed by a population of enteroendocrine cells named intestinal L-cells. Preproglucagon gene expression generally increases aborally, although relatively few species have been studied and GLP-1 or intestinal L-cell concentration have been used to assess GLP-1 secretion in addition to PCR. Importantly, preproglucagon gene expression in the ovine intestine has not been reported. The GLP-1 receptor (GLP1-R) is expressed in a wide variety of tissues, including the pancreas, liver, brain, kidneys, heart, vascular smooth muscle, adipose tissue, the stomach, gonadal tissue and the hypothalamus.

Both central and peripheral GLP-1 is secreted in response to food intake, with a peak in secretion occurring approximately 15 minutes after ingestion (Dailey & Moran, 2013). Secretion is a direct response to feeding behaviour and nutrient delivery (Faulkner & Martin, 1998) and increased secretion is associated with a positive energy balance and, subsequently, conditions favourable for reproduction. There is some indication that GLP-1 may act upon the reproductive axis, with neuroanatomical evidence of communication between GnRH neurons and GLP-1 producing neurons

(Farkas et al., 2016; C. Vastagh et al., 2021) however interventional studies which have explored this relationship directly are few. An *in vivo* study by Arbabi et al. (2021) found that microinjection of GLP-1 or Exendin-4 into the median eminence (ME) of the hypothalamus the stimulated LH secretion from the pituitary, supporting evidence GLP-1 can modulate reproductive hormones at the level of the hypothalamus, likely through stimulation of GnRH secretory terminals. Results reported by Outeirino-Iglesias et al. (2015) were contradictory, however, as although GLP-1 was associated with magnification of the preovulatory LH surge, the GLP-1 agonist Exendin-4 had an opposing effect, reducing the surge amplitude. Although Farkas et al. (2016) found that GnRH neurons expressed the GLP-1 receptor, *in situ* hybridization performed by Arbabi et al. (2021) failed to demonstrate co-expression of GLP-1 receptors on GnRH neurons.

Importantly, much of the research on whether GLP-1 influences the reproductive axis utilizes centrally administered GLP-1 or indirect evidence such as hormone or receptor expression, despite the importance of the large intestine in GLP-1 secretion. The presence of GLP-1 receptors in the ME of the hypothalamus, which sits outside the blood-brain-barrier, suggests that GLP-1 in systemic circulation may be able, even likely, to contribute to any effect seen on the hypothalamic neurons. The question, however, remains; can a peptide which originates from the distal intestine and is related to glucagon act on the hypothalamus or pituitary to promote reproduction? Confirmation of this would elucidate our understanding of the physiology underlying the relationship between metabolism and reproduction. A more thorough understanding of the communication mechanisms that exist between

energy balance and reproduction may allow these to be managed more efficiently and allow more effective feeding practices of livestock to achieve reproductive outcomes, such as more targeted nutritional flushing of ewes. The effects or climate change, with more frequent drought conditions predicted in Australia, combined with the increasing protein demand of the human population, are important motivators in better understanding how reproduction may be better manipulated by nutrition. Understanding the underlying physiology should allow a more targeted approach than the endless feed trials that currently exist in the literature. Importantly, GLP-1 and insulin response have been implicated in some of the poor fertility outcomes and disease syndromes associated with obesity in humans, including polycystic ovarian syndrome (PCOS) (Jensterle et al., 2019; Moffett & Naughton, 2020). If it can be demonstrated that GLP-1 is an important promoter of the reproductive axis it may provide a therapeutic target for treatment of these conditions.

Our hypothesis is that GLP-1 upregulates the hypothalamic-pituitary-gonadal axis of the ewe. To address our general research question of "does GLP-1 affect the reproductive axis of the ewe?" we developed five specific research questions. These are:

1) Does intravenous administration of a GLP-1 agonist affect LH and/or follicle stimulating hormone (FSH) secretion?

2) Does stage of oestrous cycle in the ewe influence the response of the reproductive axis to a peripherally administered GLP-1 agonist?

3) Does infusion of a GLP-1 agonist affect the timing of onset of the preovulatory LH surge in the ewe?

4) Is the preproglucagon gene expressed in the ovine intestine?

5) Is preproglucagon gene expression affected by stage of oestrous cycle in the ewe?

Research questions 1-3 will be explored using infusion studies, with an infusion of the GLP-1 receptor agonist Exendin-4 given intravenously and plasma LH and FSH concentrations measured to assess the response of the reproductive axis. Exendin-4 is an equipotent, full agonist for the GLP-1 receptor, with a half-life of 30 mins (Madsbad, 2016). A preliminary study has been designed with ewes in either the follicular or luteal stage of the oestrous cycle to address research question 2 and inform further study design.

Real-time PCR or “quantitative” (qPCR) will be used to address research questions 4 and 5. Samples of intestine from ewes of known oestrous cycle stage have been provided by Monash University following the conclusion of a separate experiment. The use of qPCR allows differential expression to be assessed and will allow us to determine whether expression varies along the gastrointestinal tract and/or between the luteal and the follicular phase of the oestrous cycle.

Dissertation Structure

This dissertation has been written in accordance with Charles Sturt University recommendations for a professional doctorate.

Chapter 2 consists of a review of the literature including important concepts relating to the regulation of the reproductive axis in ewes, regulation of appetite within the hypothalamus and potential signaling mechanisms between metabolism and reproduction. There is also a comprehensive review of the structure, production, secretion and metabolism and actions of GLP-1, the GLP-1 receptor and agonists before exploring what is known about GLP-1 function in ruminants and how GLP-1 may affect reproduction.

Chapter 3 consists of the preliminary study designed to explore whether a peripherally administered GLP-1 agonist would affect LH and FSH secretion in intact, cycling ewes and inform the design of a larger study. Experimental design and results are presented in the form of a prepared manuscript.

Chapter 4 consists of a paper accepted for publication in the Journal of Endocrinology entitled “The GLP-1 agonist, Exendin-4, stimulates LH secretion in female sheep”. This chapter presents the major findings of our research, including an overview of the preliminary study and PCR study. The focus of this chapter is the effect of the infusion of Exendin-4 on LH secretion in a population of ewes in the follicular phase of the oestrous cycle.

Chapter 5 presents the effect of Exendin-4 infusion on FSH secretion in ewes in the follicular phase of the oestrous cycle using data that was collected during the same experiment as described in Chapter 4.

Chapter 6 consists of the full methodology results and interpretation of our qPCR study (covered briefly in Chapter 4) performed on samples of gastrointestinal tract from intact, cycling ewes and is presented in the form of a prepared manuscript.

Chapter 7 is an exegesis which provides discussion of the results of our research as a whole, the implications of our findings and the contributions of this research to scientific literature including possible avenues for future research.

Chapter 2: Review of the Literature

Chapter 2: Review of the Literature

Introduction

The relationship that exists between nutrition, energy balance and reproduction has long been appreciated. Scholars as early as Aristotle are believed to have considered the influence of nutrition on reproduction, whilst experimental evidence for the benefits of nutritional "flushing" of ewes dates to 1899 (Clark, 1934 in Martin 2010).

One obvious reason a relationship exists between reproduction and energy balance is that the former comes at a cost to the latter (Clarke, 2014). Energy used for reproduction – the synthesis of hormones, gamete production, and reproductive behaviour- cannot be used for storage or other metabolic activities. Nutrition also has indirect effects on reproduction. In animals much is understood about the effects of poor nutrition and lean body condition, however there are still substantial knowledge gaps in how this relationship is regulated. Historically, the relationship between nutrition and reproduction in animals has been evaluated using experiments where either diet or body condition is manipulated. Some of the known effects of nutrition on reproduction are as follows:

In ruminants, feed restriction can have a marked effect on the timing of puberty. Holstein heifers growing at 1.1, 0.8 and 0.5kg/day reached puberty at 9, 11 and 16 months respectively (Chelikani et al., 2003). Similarly, sheep and goats can reach puberty as early as 5-6 months of age if well grown

(Robinson et al., 2006). However, puberty may be delayed until the following autumn if growth is restriction occurs and puberty does not occur during the first available breeding season. The importance of seasonality over nutrition is less pronounced in sheep breeds which have been selected for production rather than hardiness, such as the Merino breed (Rawlings & Bartlewski, 2007). It would appear that the "less seasonal" a breed or species is, the more important nutritional cues become (Adam, Findlay, Kyle, & Young, 1998).

Return to cyclicity post-partum is also impacted by post-partum nutrition, and body condition score. Ewes which lamb during the normal mating season will not experience a post-partum anoestrus (Robinson et al., 2006). Replacing maize grain with an equal weight of sugar beet pulp, which has considerably lower rumen bypass starch content, in a lactation diet has been found to reduce the time to cyclicity from 35±3.1 to 26±2.1 days in sheep, likely due to differences in energy availability to the animal (Mitchell et al., 2003). Similarly, beef cows in good condition at calving and receiving a diet high in metabolisable energy post-calving have a shortened anoestrus period (Wright, Rhind, Whyte, & Smith, 1992).

Nutrition can directly affect fertility through its effect on macro- and micro-nutrient supply to developing ovarian follicles, gametes and embryos (Robinson et al., 2006) and the sequestration of sex steroids in fat (Clarke, 2014). Negative energy status post-calving suppresses gonadotrophin-releasing hormone (GnRH) secretion and resumption of luteinizing hormone (LH) pulsatility in dairy cows (Roche, Mackey, & Diskin, 2000). Ovulation does not occur, despite normal ovarian follicular

growth and size and the presence of adequate gonadotrophin reserves in the pituitary (Robinson, 1996). When ovulation does proceed, the direct effect of poor nutrition may result in a small or poor-quality oocyte and a small corpus luteum (CL) due to a relative lack of substrates. Small corpora lutea from a feed-restricted animal are likely to produce less progesterone and will regress more readily than that of a well-fed animal (Robinson et al., 2006).

Poor nutrition in utero is associated with delayed puberty, lower ovulation rates and reduced litter size in adult females, presumably due to a direct effect on ovarian development as gonadotrophin function seemed unchanged (Da Silva, Aitken, Rhind, Racey, & Wallace, 2001; Rae et al., 2002; Robinson et al., 2006). Similarly, undernutrition pre-weaning can cause a reduction in litter size in sheep for three years, and may be permanent (Rhind et al., 1998; Robinson, Ashworth, Rooke, Mitchell, & McEvoy, 2006).

The increasing use of superovulation, oocyte harvest and embryo transfer has provided insight into adverse effects of a high or inappropriate plane of nutrition on fertility (Leese, 2002). Diets high in rumen degradable protein inhibit the growth and function of ovarian granulosa cells in ruminants, presumably due in part to high circulating ammonia (Robinson et al., 2006). Rapidly fermented and protein-rich diets are also associated with reduced number and quality of embryos and attachment failure in livestock species (Leese, 2002).

In addition to the direct effects of nutrition on gamete and embryo quality there is a growing body of evidence for an indirect relationship between energy status and reproduction. The evolutionary

advantage of this is easily appreciated; signaling between the energy and reproductive status of an animal provides a means to ensure parturition occurs when there is sufficient energy supply to maintain both the mother and the offspring (De Bond & Smith, 2014). In many species, seasonality is crucial in the timing of breeding and subsequent birthing period, however nutritional cues undeniably play a role in this process. Indirect effects of nutrition will be reflected by changes in hormone signaling i.e., the hypothalamic-pituitary-gonadal axis (HPG). The primary hormones involved in this feedback loop are kisspeptin, GnRH, LH and follicle stimulating hormone (FSH) from the pituitary and hormones from the gonads (e.g., oestrogen, progesterone, testosterone and inhibin). These hormones regulate follicular wave development, ovulation and ovulation rate in females and sperm production in males. Whilst it is possible a direct relationship exists, changes in these hormone levels often occurs before feed or condition is restricted to the point where substrate delivery for hormone production would be affected.

Ovulation rate of adult ruminants is affected by nutrition (Robinson et al., 2006), with ewes on a restricted diet having a significant decrease in LH concentration and frequency and FSH concentration (De Bond & Smith, 2014). Poor nutrition in ewes 6 months prior to the breeding season (i.e. late spring/early summer) reduces the number of follicles that emerge from the primordial pool. This reduces the number of follicles available for ovulation during the breeding season itself (Robinson et al., 2006). Nutritional “flushing” of ewes with high quality feed e.g., lupins for a short period prior to joining has been known to increase ovulation rate and increase the number of multiple births within a

flock. A review by Viñoles, (2003) concluded that improved feeding over a period as short as 4 days, starting 8 days before ovulation, is sufficient for the beneficial effects seen with longer periods of "flushing" e.g. 14 days. Interestingly, this effect is more pronounced in ewes which carry the Booroola fecundity gene (Robinson et al., 2006). This would suggest that nutritional supplementation of ewes allows them to meet, but not exceed, their genotypic potential. The relationship between short-term changes in feeding practices and ovulation rate indicates a signaling system must exist between energy balance, food intake and the reproductive system.

The reproductive axis, with a focus on the hypothalamus of the ewe

Before we can explore how nutritional status may influence reproduction it is important to understand the sites of possible control. Reproduction in mammals is primarily governed by positive and negative feedback between three endocrine glands: the hypothalamus, the pituitary and the gonads (ovaries or testes). This feedback loop is typically referred to as the hypothalamic-pituitary-gonadal (HPG) or reproductive axis and its function is well-preserved within mammals (Pak & Chung, 2011). The HPG axis is often thought of as a "closed" loop, however inputs relating to stress, nutrition and seasonality can interact with this axis to modify its response (Clarke & Arbabi, 2016). The hypothalamus is a highly specialized area of the brain, located on its ventral aspect between the thalamus dorsally and the pituitary gland ventrally and has distinct nuclei with functions relating to homeostasis, reproduction, behaviour and satiety (Simpson, Martin, & Bloom, 2008). The hypothalamus is the origin of GnRH secretion in mammals. GnRH is a decapeptide which stimulates the release of pituitary gonadotrophs

(LH and FSH). In sheep, the majority of GnRH neurons are located in the pre-optic area (POA) with a smaller population in the arcuate nucleus (ARC) (Lehman, Robinson, Karsch, & Silverman, 1986). GnRH neurons have exceptionally long axons which extend to the median eminence (ME), regardless of cell body origin (Pak & Chung, 2011). The neurosecretory terminals of GnRH neurons are within the ME, allowing secretion of GnRH into the hypothalamic-hypophyseal portal system. GnRH neurons are anatomically unique, with long dendrites which possess numerous spines along their length, providing multiple regulatory sides for presynaptic neurons (Pak & Chung, 2011).

The role of GnRH as the main releasing hormone that drives reproduction has led to it being considered the “master” hormone of the reproductive axis (Senger, 2012). Secretion of GnRH is pulsatile. Pulsatile secretion of GnRH is necessary for appropriate downstream gonadotrophin release, as continuous or prolonged exposure downregulates GnRH receptor expression on gonadotrophs (Pak & Chung, 2011). Phasic secretion of GnRH appears to be, at least in part, due to an intrinsic pulsatility, as GnRH neurons show episodic firing in explant cultures, despite receiving no afferent inputs (Pak & Chung, 2011). The mechanism whereby this pulsatility is communicated between GnRH neurons is uncertain. This could be achieved by dendro-dendritic connections (Campbell, Gaidamaka, Han, & Herbison, 2009), direct cellular contact between axon terminals in the ME, or paracrine signaling (Pak & Chung, 2011). Intrinsic pulsatility brings into question the existence of an external “pulse generator”. Instead, efferent signals may alter the inherent phasic nature of GnRH neurons, rather than initiate a pulse.

Recently, there have been considerable increases in our knowledge of how GnRH neurons themselves are regulated. Compared to other parts of the hypothalamus, GnRH neurons receive relatively little synaptic input (Decourt, Tillet, Caraty, Franceschini, & Briant, 2008; Lehman, Karsch, & Silverman, 1988; Scott, Rose, Gunn, & McGrath, 2018). Importantly, GnRH neurons do not express steroid receptors e.g., for oestrogen and progesterone (Clarke, 2014) despite oestrogen and progesterone having positive and negative feedback effects on GnRH secretion (Goodman, 2015). Although GnRH neurons don't express the ERα receptor, which mediates the effect of oestrogen, they do express the ERβ receptor, so oestradiol may have some effect at this level, although ERβ receptor knock-out mice remain fertile and E2 implants into the POA have little effect, so the relationship between steroid regulation of GnRH remains unclear (Hrabovszky et al., 2000; Hrabovszky et al., 2001; Scott et al 1997). Our understanding of GnRH regulation has expanded considerably with the discoveries of kisspeptin and GnIH, (also known as RFRP3) (Clarke, 2011). These peptides are now recognized as important upstream regulators of GnRH secretion and likely have a direct effect on GnRH neurons.

Kisspeptin is a 54 amino acid peptide which was found subsequent to the discovery of the GPR54 receptor, now generally termed the kisspeptin 1 (KP1R) receptor (Clarke, 2018). Kisspeptin producing cells are located in the ARC and POA in sheep, regions of the hypothalamus known to regulate reproduction. Kisspeptin cells from the POA have direct input to GnRH neurons, whilst those in the ARC may regulate GnRH secretion through an intermediary cell type or at the neurosecretory zone of the ME (Backholer et al., 2010). Kisspeptin-producing cells located in the ARC almost all co-express

neurokinin B and dynorphin and are named KNDy neurons. (Scott et al., 2018). In this way, kisspeptin and KNDy cells likely integrate and contribute to the moderating effects of stressors such as nutritional intake, seasonality, temperature on GnRH secretion (Clarke, 2011). KNDy cells are also thought to contribute to the GnRH pulse generator, with the coordinated action of neurokinin B and dynorphin resulting in pulsatile output of kisspeptin to GnRH neurons in the ARC (Lehman, Coolen, & Goodman, 2010).

Kisspeptin has a stimulatory effect on GnRH secretion and is necessary for reproduction (Clarke, 2011). Kisspeptin expression is upregulated in the ARC and POA in the follicular phase of the oestrous cycle, when GnRH secretion increases in frequency before the pre-ovulatory GnRH surge, consistent with a role in these events (Clarke, 2011). It is uncertain whether kisspeptin acts on GnRH neuron cell bodies or on the secretory terminals in the ME where GnRH is stored before release. There are no direct connections between kisspeptin neurons and GnRH terminals in the ME, so if the latter scenario is true it suggests a novel form of regulation, such as volume control or a non-neuronal intermediary such as tanycytes (Arbabi et al., 2021; Clarke, 2014).

Although the existence of an inhibitory hormone acting in concert with GnRH has long been suggested, the lack of early contenders led to the concept being largely ignored or forgotten (Clarke, 2011). The discovery in 2000 of an RF-amide peptide in the hypothalamus of Japanese quail which had a negative regulatory effect on the reproductive axis (Tsutsui et al., 2000) has changed this, although relatively little is known about the role of what was initially termed gonadotrophin inhibitory

hormone (GnIH) but has now been changed to RFRP3. The RFRP3 gene was identified in mammals in 2000, although the post-translational products differ between mammals and birds (Hinuma et al., 2000). RFRP3 neurons are concentrated in the dorsomedial nucleus and paraventricular nucleus (PVN) in sheep (Clarke et al., 2008) areas associated with regulation of energy balance and neuroendocrine hormones (eg oxytocin) respectively. RFRP3 neurons extend projections to a wide number of cells within the hypothalamus and to the amygdala and PVN, with RFRP3 immunoreactive terminals observed in the neurosecretory zone of the ME (Clarke, 2014). The RFRP3 receptor, GPR147, has been found to be expressed in the hypothalamus and pituitary in rodents and humans (Quennell, Rizwan, Relf, & Anderson, 2010; Ubuka et al., 2009). RFRP3 is known to have a direct, inhibitory effect on synthesis and secretion of both LH and FSH (Clarke et al., 2008) whilst murine cell line studies suggest it suppresses GnRH production by hypothalamic neurons (Gojska, Friedman, & Belsham, 2014).

GnRH is secreted from neurosecretory terminals of GnRH neurons in the ME, where it then enters the hypothalamo-hypohyseal portal system. GnRH then binds to gonadotroph cells to stimulate the secretion of the gonadotrophins, LH and FSH (Pak & Chung, 2011).

The gonadotrophins are members of a large family of glycoprotein hormones which consist of two polypeptide subunits (α and β) connected by an oligosaccharide chain (Pak & Chung, 2011). The α-subunit is encoded by a single gene and is common between all glycoproteins within a species. It also appears to be well conserved between mammalian species (Bousfield, Butnev, Russell Gotschall, Baker, & Moore, 1996; Pak & Chung, 2011). However the β-subunit varies for each glycoprotein

hormone and dictates its function (Pak & Chung, 2011). Gonadotrophs are a heterogenous population of cells in the anterior pituitary, some of which secrete LH, some FSH and some both (Padmanabhan, McFadden, Mauger, Karsch, & Midgley Jr, 1997). Differential secretion of LH and FSH is determined by GnRH pulsatility, with more frequent pulses (every 30-60 minutes) favoring LH secretion and a longer inter-pulse interval (every 2-4 hours) promoting FSH secretion (Burger, Haisenleder, Dalkin, & Marshall, 2004; Padmanabhan & Cardoso, 2020). Differential regulation appears to be at least in part a result of the storage of LH and FSH in discrete secretory granules within gonadotrophs (McNeilly, Crawford, Taragnat, Nicol, & McNeilly, 2003; Padmanabhan & Cardoso, 2020).

In females, LH primarily regulates steroidogenesis and is responsible for the formation of the corpora lutea following ovulation ("luteinisation") (Pak & Chung, 2011). If we consider GnRH the master hormone of reproduction then LH is the signal amplifier. There is a strong temporal relationship between GnRH and LH secretion with the pulsatile release of LH from the pituitary directly reflecting pulsatile secretion of GnRH (Clarke & Cummins, 1982). Pulsatile secretion of GnRH and therefore LH is continuous throughout the oestrous cycle of the ewe, however amplitude and frequency changes depending on whether the ewe is in the luteal or follicular phase (Rawlings & Cook, 1993). There is an inverse relationship between LH pulse amplitude and frequency; as pulse frequency increases, the amplitude decreases (Clarke, 2014). An increase in LH pulse frequency and amplitude precedes the pre-ovulatory GnRH and LH surge (Rawlings & Cook, 1993). Basal LH secretion and pulse frequency decrease during the metoestrus and dioestrus periods that follow ovulation as a result of the negative

feedback effect of progesterone from the CL on GnRH secretion. Following luteolysis and the associated decrease in progesterone concentrations, LH pulse frequency increases again before the next pre-ovulatory surge (Bartlewski, Baby, & Giffin, 2011).

Unlike the pulsatile pattern of LH secretion, FSH secretion does not directly reflect GnRH secretion (Clarke, 2011) and removal of GnRH input does not lead to an immediate cessation of FSH secretion as it does for LH (Clarke, Moore, & Veldhus, 2002). FSH is generally secreted in a passive manner in direct relation to the releasable pool of FSH in the pituitary (Clarke et al., 2002), although there is some evidence that pulsatile secretion is superimposed on basal secretion of FSH (Padmanabhan, McFadden, Mauger, Karsch, & Midgley, 1997). The relatively long half-life, high proportion of basal secretion and presence of different isoforms means pulsatile secretion of FSH is not detectable in peripheral circulation, regardless of whether it occurs or not (Padmanabhan & Cardoso, 2020). Regulation of FSH secretion appears to be multi-factorial. Gonadal steroids (e.g. oestradiol) can have a direct suppressive effect on FSH secretion (Clarke, Cummins, Crowder, & Nett, 1989). This effect is mediated in part by reduced expression of the GnRH receptor on pituitary gonadotrophs, reduced transcription of the FSH βsub unit mRNA (Bernard, Fortin, Wang, & Lamba, 2010) and post-translational modifications (Bousfield & Harvey, 2019; Padmanabhan & Cardoso, 2020). Proteins from the ovaries, namely activin, inhibin and follistatin, also play a role in regulation of FSH secretion and appear to have a direct effect at the level of the pituitary (Clarke, 2014; Padmanabhan & Cardoso, 2020). Activin has a positive effect on FSH secretion, increasing FSH mRNA expression and release

(Orisaka et al., 2021; Padmanabhan & Cardoso, 2020). Follistatin and inhibin have an opposing effect which seems to be primarily by blocking the action of activin at the level of the receptor on the gonadotroph cell (Padmanabhan & Cardoso, 2020). All three peptides are produced primarily by gonadal cells, with some production in the pituitary and hypothalamus, meaning they can have autocrine, paracrine and endocrine effects (Pangas & Rajkovic, 2015).

Interestingly, the presence of a large follicle does not prevent the emergence of a new follicular wave in ewes, even those induced by exogenous FSH part-way through the inter-wave interval (Bartlewski et al., 2011). It is not uncommon for the large follicles of both the ovulatory and the preceding follicular waves to ovulate simultaneously, particularly in prolific breeds (Bartlewski et al., 2011). This suggests ovarian feedback on FSH release may be relatively weak in sheep, contributing to the incidence of multiple ovulations in some breeds.

In sheep, there are two main periods of increased FSH release; the first at the time of the preovulatory LH surge and the second 20-36 hours later, which tends to be of longer duration (20-24hrs cf 11-12hrs) (Bartlewski et al., 2011). The first increase is thought to be due to a decrease in oestrogen and inhibin secretion from the pre-ovulatory follicle, associated with the pre-ovulatory GnRH surge (Baird, 1978; Clarke, 2018). The second is thought to reflect the removal of these inhibiting factors with the endocrine events of ovulation (Baird & McNeilly, 1981). Otherwise, FSH secretion is tightly coupled with follicular wave emergence, which typically occurs on days 0-1, 5-6

and 10-13 of the oestrous cycle (Bartlewski et al., 1999). Emergence of a follicular wave is preceded by transient increases in plasma FSH concentrations (Bartlewski et al., 2011).

The “wave like” pattern of FSH secretion results in sheep displaying a wave-like pattern of follicular development similar to cattle (and humans (Baerwald & Pierson, 2020)). There are typically three or four follicular waves per oestrous cycle during both the breeding season and seasonal anoestrus (Ginther et al., 1995; Bartlewski et al.,). Unlike in cattle, the number of follicular waves which occur per oestrous cycle does not seem to determine cycle length (Goodman, 2015). Ewes have a 16-17 day cycle, with a luteal phase typically lasting 13-14 days and 3-4 days of follicular phase, during which behavioural oestrus occurs (Bartlewski et al., 2011; Goodman 2015; Rawlings & Bartlewski, 2007)

Typically, 1-4 follicles from each wave attain the final stages of development (Bartlewski et al., 2011). Although FSH is necessary for the early stages of follicle recruitment and antral growth, final growth and development is independent of changes in FSH and instead becomes LH dependent. The earliest stages of follicle development (primordial, primary and secondary stages) occur independent of gonadotrophin effect in mammals (Orisaka et al., 2021). As follicles transition from the secondary to the pre-antral stage they gain FSH receptors and growth becomes FSH dependent (Orisaka et al., 2021). The FSH receptor (FSHR) is expressed on the granulosa cells of pre-antral follicles and promotes proliferation and differentiation through a number of cellular pathways. Apoptosis of granulosa cells is suppressed by FSH, meaning acquisition of FSHR is a key determinant in whether follicles undergo atresia or are selected and become dominant (Orisaka et al., 2021). Pre-antral follicles also develop a

thecal cell layer which expresses the LH receptor (LHR) in response to stimulation from FSH, allowing steroid synthesis. Androgens produced by thecal cells are converted to oestradiol by FSH-induced aromatase in granulosa cells, promoting further follicular growth and providing feedback to the pituitary (*vide supra*) (Orisaka et al., 2021). As follicle selection progresses, FSHR expression is suppressed and LHR expression increases significantly, signaling the LH dependent maturation phase of the follicle. The LHR is expressed only on thecal and mural granulosa cells of antral (final stage) follicles (Orisaka et al., 2021).

The growing antral follicle(s) secretes increasing levels of oestradiol 17β (E2) and triggers a change in the hypothalamus and pituitary whereby E2 no longer exerts negative feedback but instead has a positive feedback effect and triggers the pre-ovulatory GnRH and LH surge (Clarke, 2018). The exact mechanisms which underpin this switch are not yet understood, although it is clear that there are significant differences between sheep, rodents and non-human primates. For instance, increased progesterone will initiate an ovulatory surge in oestrogen-primed rodents, but is ineffective in ruminants (Clarke, 2018). Indeed, the pre-ovulatory GnRH surge will not proceed in the presence of progesterone in sheep, highlighting the risk of extrapolating findings based on rodent reproductive physiology. In sheep, E2 concentrations must increase acutely to a critical level in the absence of progesterone and remain there for a few hours to initiate positive feedback (Clarke, 2018). By contrast, ongoing exposure to E2 results in downregulation of GnRH release.

Positive feedback in the hypothalamus appears to be mediated by the ERα receptor, an interesting finding given it is not expressed on GnRH neurons (Wintermantel et al., 2006). Instead, it seems that GnRH neurons in the POA receive input from neurons that do express the ERα and that this may involve interneurons or other intermediary cells (Clarke, 2018). Noradrenergic neurons from the brainstem, glutamate and GABA neurons and kisspeptin neurons particularly seem to mediate the change from negative to positive feedback in response to a threshold concentration of E2 (Clarke, 2018). In sheep, KNDy neurons in the ARC are activated within an hour of this stimulus, whilst kisspeptin neurons in the POA are activated at the time of the surge. The kisspeptin neurons in the ARC are known to be involved in negative feedback of E2 in sheep, so it seems that these cells are able to change function between negative and positive feedback control (Clarke, 2018). It now seems that kisspeptin neurons of the ARC initiate the positive feedback of E2 (Smith et al., 2011) and are facilitated by the kisspeptin neurons of the POA (Clarke, 2014).

Positive feedback of E2 in the hypothalamus leads to GnRH pulsatility increasing to a frequency whereby pulses start to build on one another, leading to a “surge” (Fig 2.1)

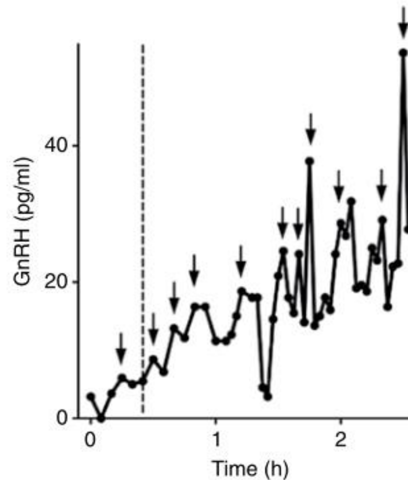

Figure 2.1 Rapid sampling identifies GnRH pulses at the start of an oestrogen-induced surge in the ovariectomized ewe. Note that the levels for identified pulses (arrows) do not return to starting values prior to the next pulse occurring; this is a surge-generating mechanism at the level of GnRH secretion. From Clarke (1993).

At the same time, increased plasma concentrations of E2 increase expression of the GnRH receptor on gonadotrophs, increase expression of gonadotrophin β subunit expression and mobilization of secretory granules containing LH (Clarke, 2018; Tobin, Pompolo, & Clarke, 2001). In this way, E2 priming of gonadotrophs (with increased GnRH pulse frequency) leads to them becoming both more sensitive to stimulation from the GnRH surge and better able to respond. The GnRH surge results in secretion of at least 95% of LH from gonadotrophs, meaning amplitude of the LH surge is dependent on LH reserves, rather than a direct reflection of the size and duration of the GnRH surge (Clarke, 2018).

The presence of LHR on mature theca and granulosa cells (see above) allow mature follicles to respond to the LH surge by ovulating and undergoing luteinization (Senger, 2012). The LH surge acts on the LHR to induce expression of a number of genes, including epidermal growth factor-like (EGF-like) factors, prostaglandin synthase, chemokines and cytokines (Goodman, 2015). These compounds work in concert to thin the thecal cell layer and cause hyperemia and dilation of the vascular network in the theca externa. Immune cells are recruited to the area and the basal lamina of the ovarian surface epithelium loosens and allows the release of the expanded cumulus cell-oocyte complex into the peritoneal cavity where it is then "captured" by the fimbrae of the uterine tubule (ovulation) (Goodman, 2015; Senger, 2012). Ovulation occurs 24-30 hours (typically 22-26 hours) after the LH surge in ewes (Goodman, 2015).

Once ovulation has occurred the process of luteinization can begin. The LH surge and EGF-like factors which result in ovulation also induce genes that regulate the process of luteinization and end the expression of genes regulated by FSH. The formation of a corpus luteum is the result of marked neovascularization of the granulosa cell layer and irreversible differentiation of granulosa cells to luteal cells. These cells have increased mitochondrial growth and activity and are capable of enhanced steroidogenesis (Goodman, 2015), becoming the source of progesterone secretion which dominates the luteal phase of the oestrous cycle.

The luteal phase lasts for 13-14 days in the ewe (Goodman, 2015). Rising progesterone secretion from the CL over the first 5 days of the luteal phase acts to "prime" the endometrium to begin episodic

secretion of prostaglandin F2α (PGF2α) 7-8 days later. The CL thereby programs its own demise. PGF2α acts to increase luteal blood flow, decrease P4 production and stimulate luteal secretion of oxytocin (Goodman, 2015). Luteal oxytocin seems to increase PGF2α in a positive feedback loop initiated by pituitary oxytocin secretion, whereby upregulation of endometrial oxytocin receptors induces pulsatile PGF2α release, which stimulates luteal oxytocin release and increased secretion of PGF2α. Activation of oxytocin receptors on small luteal cells induces apoptosis, whilst increased intracellular calcium in large luteal cells also induces apoptosis (Goodman, 2015). In this way, secretion of PGF2α from the endometrium leads to physiological and structural demise of the CL or "luteolysis", and the end of the luteal phase.

This cycle typically continues, unless interrupted by pregnancy, throughout the breeding season.

It is important to note that sheep display seasonality, with the degree determined largely by breed (Bartlewski et al., 2011; Rawlings & Bartlewski, 2007). Breeds which developed nearer the poles are more seasonal than those closer to the equator (Rosa & Bryant, 2003). Breeds from temperate climes, such as the Australian Merino, have a short period of anoestrus and approximately 5% will ovulate spontaneously year-round (Rosa & Bryant, 2003). The sheep is a "short day" breeder, with breeding activity greatest when day length is short (Edmondson, Roberts, Baird, Bychawski, & Pugh, 2012). With a gestation length of approximately 147 days lambs are born in the spring, when nutrient supply for both dam and offspring are good, and climatic conditions favor survival (Rosa & Bryant, 2003). Interestingly, the metabolic function of sheep also displays seasonality, with appetite decreased

during the short-day period, coincident with the breading season (Clarke, 2014). Day length also seems to influence energy expenditure and adiposity in sheep (Clarke, 2014). Metabolic seasonality seems to be regulated in part by altered NPY and POMC expression with leptin, ghrelin and KNDy neurons also likely involved (Clarke, 2014).

Although photoperiod is the most significant regulator of the annual rhythm of cyclicity, it needs to be remembered that other factors such as exposure to males, ambient temperature and nutrition, particularly, can all act to mediate the oestrous cycle in the ewe.

Regulation of appetite

The ability of organisms to maintain appropriate body weight and adiposity whilst consuming and storing enough energy to allow necessary functions such as thermoregulation and reproduction to proceed unimpeded is regulated by a complex interplay of hormonal, chemical and neuronal networks, as reviewed in Simpson et al. (2008) and Ahima and Antwi (2008). Generally, organisms have evolved to conserve energy, as unrestricted access to food is an uncommon occurrence (Suzuki, Simpson, Minnion, Shillito, & Bloom, 2010). The current human obesity epidemic and slew of related conditions highlights the wide-ranging effect that dysfunction of this axis can have on populations. When considering regulation of energy balance there are several key concepts and points of control we need to consider. Firstly, there needs to be mechanisms in place to detect the amount of stored energy, generally as adipose tissue, and the amount of readily available energy; this is glucose in most species. Secondly, there needs to be signals that promote feeding when energy balance is at a

potential deficit ("hunger"), and an "off switch" to stop feeding activity when energy needs are likely to have been met ("satiety"). An anorexigen signals appetite suppression, whilst an orexigen will promote feeding behaviour (Clarke, 2014). Regulation of energy balance is mediated primarily by the hypothalamus and it is well placed to do so, receiving input from both higher centers of the brain and from the periphery via the incomplete blood-brain barrier which exists at the ME. Early lesion and electrostimulation studies demonstrated that the lateral hypothalamic area (LHA) was important in feeding behaviour (Anand & Brobeck, 1951) whilst the ventromedial nucleus (VMN) was involved in restriction of feeding (Hetherington and Ranson 1940, in Sohn (2015), leading to the "dual center" hypothesis whereby the LHA functions as a "hunger" center whilst the VMN is a "satiety" center (Simpson et al., 2008). We now know the relationship is much more complex than this, with a number of hypothalamic nuclei involved (Simpson et al., 2008).

In addition to its role in reproduction, the ARC is recognized as a key area in the regulation of energy metabolism (Ahima & Antwi, 2008; Simpson et al., 2008), an interesting anatomical relationship which hints at how well these two axes are intertwined. The location of the ARC near the permeable blood-brain barrier of the ME places it in a location conducive to sensing and integrating peripheral signals of energy metabolism with input from higher centers in the brain (Simpson et al., 2008). The ARC has two subpopulations of neurons which are integral to appetite regulation; pro-opiomelanocortin expressing (POMC) neurons and neuropeptide Y (NPY) neurons. POMC are located in the lateral ARC. POMC neurons in the ARC also express cocaine-and amphetamine-related transcript (CART).

Stimulation of these neurons has an anorexigenic effect (Simpson et al., 2008; Sohn, 2015). Excitation of POMC/CART neurons may also activate melanocortin pathways that increase energy expenditure (Sohn, 2015). The medial ARC houses a subpopulation of neurons considered key in orexigenic pathways; the NPY neurons (Sohn, 2015). An estimated 95% of this subpopulation co-express agouti-related peptide (AgRP), so they are commonly referred to as NPY/AgRP neurons (Simpson et al., 2008). NPY/AgRP release the inhibitory neurotransmitter GABA in addition to NPY and AgRP. It is not clear how the orexigenic effect of NPY/AgRP neurons is mediated, however the inhibitory effect of GABA on anorexigenic centers in the CNS, antagonism of MC4R (see above) by AgRP and metabolic effects of NPY on Y receptors are likely involved in a complex relationship (Sohn, 2015; Suzuki et al., 2010). The POMC/CART neurons of the ARC themselves receive input from NPY/AgRP neurons, providing local feedback on appetite control within the ARC (Cowley et al., 2001).

The POMC/CART and NPY/AgRP neurons provide downstream input to other hypothalamic nuclei. These are the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventromedial nucleus (VMN) and dorsomedial nucleus (DMN) and parabrachial nucleus (PBN).

The PVN receives neuronal input from NPY/AgRP and POMC/CART neurons of the ARC and contains thyroid releasing hormone (TRH) and corticotrophin releasing hormone (CRH) secreting neurons, meaning it can have downstream effects on metabolism and energy balance as well as integrating information from the ARC. TRH and CRH themselves both have an anorectic effect (Simpson et al., 2008).

The LHA is an important orexigenic center, with extensive NPY/AgRP immunoreactive terminals (Simpson et al., 2008) and contains the orexigenic neuropeptides melanin concentrating hormone (MCH) and orexin (also called hypocretin) (Simpson et al., 2008; Sternson & Eiselt, 2017) and is likely involved in food-seeking behavior, taste and positive reinforcement behaviours (Sternson & Eiselt, 2017).

The VMN is an important anorexigenic center, with lesions in this area leading to rapid onset hyperphagia and obesity (Simpson et al., 2008). In addition to receiving input from the ARC it has a large population of glucoresponsive neurons that allow it to respond to feeding-related stimuli such as glucose, dopamine and serotonin, independently (Simpson et al., 2008).

The DMN is another anorexigenic center which receives input from the ARC and has projections to TRH neurons in the PVN (Simpson et al., 2008). Lastly, the PBN has a population of calcitonin gene related peptide (CGRP) neurons which potently suppress eating when activated (Sternson & Eiselt, 2017)

Although the hypothalamus is essential in the integration and regulation of appetite-related signals, other areas of the brain are involved. The brainstem, in particular, has an important role in energy metabolism and there are extensive, reciprocal projections between the brainstem and hypothalamus (Horst, De Boer, Luiten, & Van Willigen, 1989). Afferent fibres of the vagus nerve project onto the brainstem and provide a neuroanatomical link between the GIT and brain. Vagus nerve inputs onto an area of the brainstem termed the dorsal vagal complex (DVC), which consists of the dorsal motor

nucleus, the area postrema and the sensory nucleus of the tractus solitarius (NTS) (Chaudhri, Wynne, & Bloom, 2008). The DVC is thereby able to interpret and relay signals from the periphery to the hypothalamus (Simpson et al., 2008). There is also a population of POMC neurons in the NTS (Simpson et al., 2008). Interestingly, the area postrema has a permeable blood-brain barrier, similar to the ME, providing an avenue by which the brainstem can sense and interpret peripheral energy substrates (Simpson et al., 2008).

Signaling between nutrition and reproduction

The ability of nutritional changes and status to affect reproduction indirectly, such as length of anoestrus, onset of puberty and ovulation rate, supports the existence of a signaling system between the two. Chronic negative energy balance will suppress HPG axis function (Dupont, Reverchon, Bertoldo, & Froment, 2014; Henry et al., 2004; Heppner et al., 2017). There is also anatomical evidence for such a relationship; interconnections exist between the neural elements that control metabolic function and those that control reproduction within the brain (Clarke, 2014). Feed restriction in ewes compromises gonadotroph function, with altered LH pulsatility and FSH secretion and enhanced negative feedback of LH in lean ewes (Renquist, Calvert, Adams, & Adams, 2008; Thomas et al., 1990). This inhibitory effect is likely mediated by NPY/AgRP neurons, which can block both pulsatile GnRH/LH secretion and the pre-ovulatory surge (Clarke, Backholer, & Tilbrook, 2005). Interestingly, the expression of the MCR3 is known to be high in the ARC and has recently been linked

to timing of onset of puberty in mice and humans, suggesting it may be the pathway responsible for some of the effects of NPY/AgRP neurons (Lam et al., 2021).

Unlike the orexigenic effect of NPY which is via the Y1 receptor, suppression of reproduction is via the Y2 receptor in sheep (Clarke, 2014). POMC activity also seems to affect reproduction, with central administration stimulating LH secretion. Another post-translational product of POMC, the opioid peptide β-endorphin is thought to have an inhibitory effect on GnRH secretion (Horton, Cummins, & Clarke, 1987). Orexin neurons in the DMN project to GnRH neurons and may act as an intermediary in POMC regulation of GnRH secretion (Backholer et al., 2010; Iqbal, Pompolo, Sakurai, & Clarke, 2001). Although ORX inhibits reproduction (Furuta, Funabashi, & Kimura, 2002; Tamura, Irahara, Tezuka, Kiyokawa, & Aono, 1999) there is some conflicting evidence of this effect (Small et al., 2003). Going against the general rule that orexigens have an inhibitory effect on reproduction, MCH appears to be stimulatory, with microinjection into the POA stimulating LH secretion (Murray et al., 2006). Expression of ERα in the POA is altered in the POA and VMN in lean, OVX ewes undergoing feed restriction (Hileman, Lubbers, Jansen, & Lehman, 1999). KNDy neurons seem likely to integrate and regulate the inhibitory and stimulatory effects of metabolic signals on reproduction within the hypothalamus. Interestingly, the ability of RFRP3 to stimulate food intake in sheep (Clarke et al., 2012) hints at a reciprocal relationship in hypothalamic control of reproduction and appetite.

Table 2.1 Neurons and neurotransmitters related to appetite and effect on the reproductive axis

Neuron/neurotransmitter	Effect on appetite	Effect on reproduction
POMC/CART	Anorexigen	Stimulatory. Likely via kiss upregulation (De Bond & Smith, 2014)
NPY/AgRP	Orexigen	Inhibitory. Decreases GnRH/LH release and can block pre-ovulatory surge (Clarke et al., 2005)
Beta endorphin	Anorexigen (post-translational product of POMC)	Inhibitory. Decreases GnRH secretion (Horton et al., 1987)
MCH	Orexigen	Unclear. Effect likely affected by steroid status and other factors (Naufahu, Cunliffe, & Murray, 2013)
Orexin/hypocretin	Orexigen	Inhibitory. Decreases LH secretion, likely mediated by KnDY neurons (Hosseini & Khazali, 2018)
GnIH/RFRP3	Orexigen	Inhibitory. Decreases GnRH/LH secretion (Clarke, 2011)
TRH	Anorexigen	Stimulatory. Likely multi-factorial and PRL-mediated (Silva, Ocarino, & Serakides, 2018)
CRH	Anorexigen	Unclear. Regulatory role, likely steroid mediated. (Ciechanowska et al., 2011)

Metabolic state also appears to alter the clearance of some hormones, with E2 clearance from plasma reduced in lean sheep (Renquist et al., 2008). Interestingly, blood-brain-barrier permeability changes with metabolic state, which could indicate the effect of a peripheral hormones is altered by positive or negative energy balance (Langlet, Mullier, Bouret, Prevot, & Dehouck, 2013).

The peripheral metabolites and hormones which regulate energy homeostasis are numerous and increasing. It is also unknown whether signaling between metabolic status and reproduction occurs centrally, at the level of the hypothalamus, the pituitary or the gonad. There are also many peripheral

hormones involved in energy regulation which may contribute to control of reproduction through either central effects or direct effects on the gonad and gametes. Given the complexity of energy homeostasis, multi-tiered nature of the reproductive axis and the number of hormones and metabolites involved it seems most likely that a complex relationship exists, with multiple points of feedback and control, rather than a simple, negative feedback mechanism. A better understanding of this relationship will allow targeted manipulation to improve reproductive efficiency in domestic animals.

The gut-reproduction axis

There are more than 100 bioactive peptides produced in the GIT from more than 30 gut hormone genes, all of which have potential effects on appetite (Suzuki et al., 2010). In addition, virtually every neurotransmitter and neuropeptide in the brain can have some effect on food intake and/or energy expenditure (Clarke, 2014). Further, the direct products of digestion eg glucose and amino acids, can themselves affect energy metabolism both directly and indirectly through hormones such as insulin and glucagon. The number of potential substrates and pathways involved highlights the complex nature of energy metabolism. However, there are a number of hormones whose role in energy balance is becoming better understood. It is beyond the scope of this paper to examine these in detail and the reader is instead directed to reviews by Huda, Wilding, and Pinkney (2006), Sandoval and D'Alessio (2015) and Comninos et al. (2014). However, the gut peptides are worth mentioning briefly due to their relationship with GLP-1 and potential to interact with the HPG axis.

The gut peptides are a group of hormones released from the gastrointestinal tract and includes ghrelin, cholecystokinin (CCK), oxyntomodulin (OXM), peptide YY (PYY) and glucagon-like peptides 1 and 2 (GLP-1 and GLP-2).

Table 2.2 Peripheral appetite-related hormones and their effect on the reproductive axis

Hormone/peptide	Effect on appetite	Primary site of production	Reproductive effect
Ghrelin	Orexigen	Fundus of the stomach	Inhibitory: decreases LH concentration and pulse frequency, inhibitory effects on CL function (Clarke, 2014)
PYY	Anorexigen	Small intestine	Unclear. Stimulates gonadotrophin secretion in some studies, reduces in others (Izzi-Engbeaya et al., 2020)
CCK	Anorexigen	Small intestine	Unreported
OXM	Anorexigen	Small intestine	Unreported
GLP-1	Anorexigen	L-cells of small and large intestine	Stimulatory. Likely to increase GnRH/LH secretion
GLP-2	Anorexigen	L-cells of small and large intestine	Unreported
Leptin	Anorexigen	Adipose tissue	Stimulatory. Threshold required for HPG (particularly gonadotroph) function (Comninos et al., 2014)
PP	Anorexigen	Pancreas	Unreported.
PYY	Anorexigen	L-cells of small and large intestine	No known effect (Izzi-Engbeaya, Jones, Crustna, Machenahalli, Papadopoulou, Modi, Panayi, et al., 2020)
Insulin	Anorexigen	Pancreas	Stimulatory. Increases GnRH/LH secretion (Sliwowska et al., 2014)
Glucagon	Anorexigen	Pancreas	No known effect (Izzi-Engbeaya, Jones, Crustna, Machenahalli, Papadopoulou, Modi, Starikova, et al., 2020)
GIP	No known effect on appetite	Intestinal K-cells	Unclear. Appears to be inhibitory (Moffett & Naughton, 2020).

Ghrelin is a peptide produced by the monogastric stomach (abomasum in the ruminant) and is a relatively recent discovery (Hayashida et al., 2001; Kojima et al., 1999). It is a potent orexigen which acts at least in part via the vagus nerve to stimulate food intake (Clarke, 2014; Crespo, Cachero, Jimenez, Barrios, & Ferreiro, 2014; Huda et al., 2006). It is the only peripheral hormone known to stimulate hunger. Ghrelin immunoreactive neurons have also been found adjacent to the third ventricle, in the region of the VMN, DMN, PVN and ARC (Huda et al., 2006). Its effect is mediated by stimulation of NPY/AgRP neurons, whilst ghrelin-expressing neurons have terminals on NPY/AgRP, POMC/CART and CRH neurons and orexin fibers in the LHA (Toshinai et al., 2003). Ghrelin has been demonstrated to affect reproductive function by suppression of the HPG axis at the level of the hypothalamus, although ghrelin receptors have not yet been found on kisspeptin or GnRH cells, suggesting an indirect relationship perhaps involving NPY neurons or the melanocortin 3 or 4 receptor (MCR3/4) pathway (Clarke, 2014; Huda et al., 2006, Smith, Woodside, & Abizaid, 2022). Ghrelin is known to suppress pulsatile LH secretion in sheep (Iqbal et al., 2001). It seems likely this is an indirect effect, perhaps mediated by KNDy neurons and the MCR3/4 pathway, which has recently been shown to have reproductive effects in humans (Lam et al., 2021).

Peptide YY was first isolated by Tatemoto (1982) in 1980. It is a 36-amino-acid peptide secreted from intestinal L cells in response to nutrients in the gastrointestinal tract via a neural reflex which may be vagally mediated (Huda et al., 2006). Peptide YY inhibits fluid and electrolyte secretion in the small intestine. It slows gastric emptying and intestinal transit and may act as an “ileal brake” (Huda et al.,

2006). Peripheral PYY acts centrally to reduce food intake. The exact mechanism of action is unclear, however it seems that PYY acts on the Y2 receptors NPY/AgRP neurons in the ARC, thereby increasing reproductive activity (Batterham et al., 2002). The vagus and brainstem also seem to be involved, as the anorexigenic effect of peripheral PYY is abolished if this pathway is damaged (Abbott et al., 2005). Interestingly, the effects of central administration of PYY are dependent on which part of the brain it is acting upon (Huda et al., 2006). There is no known effect of PYY on the HPG axis (Izzi-Engbeaya, Jones, Crustna, Machenahalli, Papadopoulou, Modi, Panayi, et al., 2020).

Oxyntomodulin (OXM) was isolated in the early 1980's and named for its inhibitory effect on the oxyntic glands of the stomach (Huda et al., 2006). It is produced by post-translational processing from the preproglucagon gene. Oxyntomodulin is released in response to food intake and levels stay elevated for several hours. It is anorexigenic, acting on the ARC, and also inhibits gastric emptying and gastric secretion (Huda et al., 2006; Simpson et al., 2008). It is a weak agonist for the GLP-1 receptor, but more specific receptors may be involved in its mechanism of action (Huda et al., 2006). The effect of OXM on reproduction has not yet been reported.

Post-translational processing of the preproglucagon gene also results in production of the hormone glucagon-like peptide 1 (GLP-1). Glucagon-like peptide 1 is the subject of our research and will be the focus of the rest of this review.

Glucagon-like peptide 1

Glucagon-like peptide 1 is a 30 amino acid peptide with 50% structural homology to glucagon (Huda et al., 2006; Sekar, 2017). The discovery of GLP-1, like all gut hormones, can trace its origin to the work of Bayliss and Starling in 1902. They observed that acid in the duodenum stimulated the flow of pancreatic juices in the absence of pancreatic innervation, providing the first proof of 'chemical sympathy' between organs, and the concept of hormones (Dockray, 2014). Other gut hormones were discovered over the next 70 years, although it wasn't until the preproglucagon gene was sequenced in the early 80s that the peptide GLP-1 was discovered (Bell et al., 1983a, Bell, Santerre & Mullenbach 1983b).

L cells and the preproglucagon gene

The preproglucagon gene is expressed in pancreatic islet (A-cells) and a population of cells in the intestinal mucosa known as enteroendocrine or L-cells, so named for the "large" secretory granules they possess (Paternoster & Falasca, 2018). It has been known since the late 60's that a population of cells existed within intestinal mucosa which resembled the glucagon-producing pancreatic A-cells. These intestinal endocrine cells could be stained using glucagon antibodies, however their morphology differed enough that they were instead designated "L-cells" (Holst, 2007). Intestinal L-cells are an "open-type endocrine cell with a slender triangular form with the base resting on the basal lamina and a long, cytoplasmic process reaching into the gut lumen" (Holst, 2007). Intestinal L-cells are located throughout the intestinal tract, although in most species their density is greatest in

the ileum, followed by the colon. As molecular studies confirmed the structure of mRNA and DNA that encodes glucagon it became evident that the same gene encoded a large “proglucagon” fragment containing two glucagon-like sequences - termed glucagon-like peptides 1 and 2. The structure of preproglucagon gene was deduced from the sequence of hamster cDNA and the human preproglucagon gene (Bell et al., 1983a, Bell, Santerre & Mullenbach 1983b). A single gene encodes for preproglucagon and more recent studies suggest this gene is well-preserved across vertebrate species, a feature often associated with genes with biologically important downstream products (Holst, 2007; Irwin, 2001, 2020). Phylogenetic studies suggest the sequences within the preproglucagon gene originated prior to vertebrate diversification more than 500 million years ago (Irwin, 2001). Where changes in the preproglucagon gene have occurred it has often reflected a change in function e.g. from a glucagon-like hormone in fish to an incretin in mammals (Irwin, 2001). In mammals, the glucagon, GLP-1 and GLP-2 sequences are encoded by separate exons, suggesting that the three peptides originated by triplication of an exon that encoded a glucagon-like sequence. Evolution of the gene in mammals has been slow relative to fish, birds and the evolution of GLP-2, suggesting it has long had biological importance in mammals (Irwin, 2001). Amazingly, the GLP-1 sequence is preserved in 100% of all mammalian species where it has been studied (Kieffer & Habener, 1999). Although the preproglucagon gene is expressed in both pancreatic A-cells and intestinal L-cells, resulting in the same mRNA sequence, there are differences in transcription of the gene. In the intestine, the transcription factor pax6 is essential for expression of the preproglucagon

gene. Beta catenin and transcription factor 4 also mediate expression in intestinal, but not pancreatic cells (Holst, 2007). Interestingly, differences in transcription may influence plasma levels of GLP-1 and susceptibility to disease (Grant et al., 2006, in Holst 2007). As transcription of the same gene sequence can result in different products depending on cell type, post-translational processing must occur.

GLP-1 production: Post-translational processing of the preproglucagon gene

The product produced by the preproglucagon gene sequence is dependent on post-translational changes by prohormone convertases (Sandoval & D'Alessio, 2015). In addition to glucagon, GLP-1 and GLP-2, the preproglucagon gene produces oxyntomodulin, glicentin peptide and two intervening peptides of uncertain significance (Huda et al., 2006; Sandoval & D'Alessio, 2015). Processing in intestinal L-cells is dependent on the co-expressed prohormone convertase (Psck) 1/3 (Holst, 2007; Ugleholdt et al., 2004). The Psck 1/3 pathway results in the formation of GLP-1, GLP-2, oxyntomodulin, glicentin and intervening peptide 2 (IP2). By comparison, the products of the Psck 2 pathway, which is dominant in pancreatic A-cells, are glucagon, glicentin-related pancreatic peptide, intervening peptide 1 and the major proglucagon fragment. Experiments in which Psck 1/3 activity was increased in A-cells with the use of an adenovirus, their production of GLP-1 increased (Wideman et al., 2006).

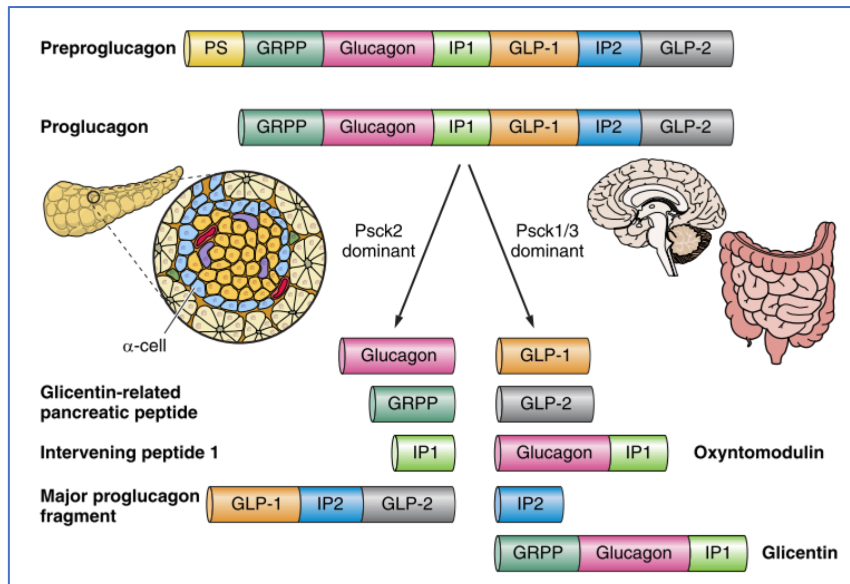

Figure 1. Posttranslational processing of preproglucagon. From Sandoval & D'Alessio (2015)

Preproglucagon and GLP-1 expression in the CNS

The preproglucagon gene is also expressed within the central nervous system. Within the CNS, preproglucagon translation is Psck 1/3 dominant, similar to translation within the intestine, and produces oxyntomodulin, GLP-1, GLP-2, IP 2 and glicentin (Holst, 2007). Recent studies in rodents have used immunohistochemistry and in-situ hybridization to confirm the expression of GLP-1 by neurons and the pattern of distribution of neuronal elements (e.g. axons) to better understand the role of GLP-1 (Farkas et al., 2016; Heppner et al., 2017). Glucagon-like peptide 1 producing neurons are located in the brainstem (Heppner et al., 2017; Mercer, Moar, Findlay, Hoggard, & Adam, 1998). Specifically, GLP-1 is expressed by neurons of the nucleus of the solitary tract (NTS) of the brainstem in rats and primates. Efferent nerve fibres from these neurons project to various parts of the brain, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), supraoptic nucleus (SON),

hypothalamus and NTS itself (Dailey & Moran, 2013; Holst, 2007; Larsen, Tang-Christensen, Holst, & Orskov, 1997; Mercer et al., 1998).

Secretion of GLP-1

Glucagon-like peptide 1 is released after eating, in a biphasic pattern; with an early peak approximately 15 minutes after eating and a later, larger peak when ingesta reaches the small intestine (Dailey & Moran, 2013; Orskov, Wettergren, & Holst, 1996). The initial rise appears to involve a neuroendocrine loop where nutrients in the stomach or proximal intestine stimulate the release of hormones that act via the vagus nerve to stimulate GLP-1 secretion from intestinal L cells. The enteric nervous system may also be involved in this early rise (Dailey & Moran, 2013). The later, larger peak is associated with nutrients in the intestine being sensed by intestinal L-cells (Dailey & Moran, 2013). Intestinal L-cells have nutrient-sensing projections which project into the intestinal lumen and secrete GLP-1 in proportion to the energy content of ingesta (Faulkner & Martin, 1998; Huda et al., 2006). Glucose and lipids are the principal nutrients which regulate GLP-1 release in simple stomached animals, whilst short chain fatty acids (e.g. propionate and butyrate) appear to be more important in ruminants (Faulkner & Martin, 1997; Fukumori, Mita, Sugino, Obitsu, & Taniguchi, 2012).

Similar to peripheral cells, GLP-1 producing neurons are activated in response to nutrient ingestion (Kreisler, Davis, & Rinaman, 2014).

Metabolism of GLP-1

Glucagon-like peptide 1 is rapidly degraded by the catalytic enzyme dipeptidyl peptidase IV (DPP IV). Dipeptidyl peptidase IV cleaves two NH_2-termnial amino acids, generating GLP-1 9-36 amide or GLP-1 (9-37), an inactive metabolite which may act as a competitive receptor antagonist, although this is yet to be supported by *in vivo* studies (Holst, 2007). Dipeptidyl peptidase IV is a widely expressed transmembrane glycoprotein that cleaves N-terminal dipeptides from a number of substrates, including hormones, neuropeptides, chemokines and growth factors (Lamers et al., 2011). It is expressed on the enterocyte brush border and on endothelial cells of capillary walls, including those lining the lamina propria (Holst, 2007). Accordingly, GLP-1 is rapidly degraded as it diffuses through the lamina propria into the circulatory system (Dailey & Moran, 2013). It's estimated that only 15-25% of secreted GLP-1 leaves the intestine via the hepatic portal vein in an active form (Holst & Deacon, 2005; Kieffer, McIntosh, & Pederson, 1995). Glucagon-like peptide 1 is further degraded by DPP IV in the liver. It is estimated that only 10-15% of all secreted GLP-1 reaches systemic circulation in the active form GLP-1 (7-36). In addition to transmembrane DPP IV there is a soluble form found in plasma and other body fluids which lacks the cytoplasmic tail and transmembrane region. Although some soluble DPP IV likely originates from inflammatory cells it has now been demonstrated that the likely source is adipocytes (Lamers et al., 2011). Expression of DPP IV has been found to increase when adipose tissue, particularly visceral adipose, stores increase, increasing GLP-1 metabolism and potentially contributing to insulin resistance and metabolic syndrome (Lamers et al., 2011; Sell et al., 2013). The rapid and widespread metabolism of GLP-1 in circulation results in a plasma half-life of 1-2

minutes. The inactive metabolite, GLP-1 (9-37) is also rapidly cleared by the kidneys, with a half-life of 4-5 minutes. In addition to DPP IV, neutral endopeptidase 24,11 has been shown to enhance degradation of GLP-1 (Plamboeck, Hoist, Carr, & Deacon, 2005). The rapid metabolism and short half-life of GLP-1 results in low plasma levels and has led to some questioning whether neural involvement, rather than a sole endocrine action, accounts for at least some of its effects. It is possible the GLP-1 that enters the blood stream is acting on vagal efferent neurons in the lamina propria or the enteric nervous system before entering capillaries. Another possibility is that degrading enzymes quickly become saturated, allowing GLP-1 to persist in the general circulation and exert its effect (Dailey & Moran, 2013).

GLP-1 receptor

The GLP-1 receptor (GLP1R) is a class 2, G-protein coupled receptor that has been found in pancreas, liver, brain, kidneys, heart, vascular smooth muscle, adipose tissue and the stomach (Dailey & Moran, 2013; Holst, 2007; Nagell, Wettergren, Orskov, & Holst, 2006). Receptors are on the cell surface and linked to G proteins, which stimulate cAMP production. Receptor binding is therefore likely to have a stimulatory effect, although its function at different sites is not always known (Faulkner & Martin, 1999; Holst, 2007). Within the brain, the GLP-1 receptor has been found to be abundant within the ARC, PVN and VMN, regions of the hypothalamus involved in metabolism, appetite regulation and reproduction (Crespo et al., 2014; Larsen et al., 1997). Although GLP-1 can be centrally produced, the presence of a dense population of GLP1R on the median eminence of the hypothalamus, which sits

outside the blood-brain-barrier, suggest peripherally produced GLP-1 may produce a central effect (Larsen et al., 1997). Recently, qPCR has been used to demonstrate the presence of GLP1R expression on GnRH cells of the hypothalamus (Farkas et al., 2016) although this has not been corroborated with *in situ* hybridization (Arbabi et al., 2021). An earlier study by Outeirino-Iglesias et al. (2015) found that GLP1R is also expressed in the pituitary gland (or adenohypophysis) and ovary, a tantalizing finding that suggests GLP-1 may have a role in regulation of the reproductive axis. This is further supported by the finding that GLP-1R is expressed in testicular and ovarian tissues (Caltabiano et al., 2020; Khan et al., 2022; Zhu, 2019).

The failure of some receptor antagonists to block all functions of GLP-1 has led to some speculation that a second receptor may exist (Holst, 2007). It is also possible that receptor location varies amongst species, accounting for some of the differences seen in experimental studies. Similar to the preproglucagon and GLP-1 sequence, the GLP1R gene sequence is well conserved across species and appears to have originated early in vertebrate evolution (Irwin, 2020).

GLP-1 actions

Glucagon-like peptide 1 has a range of effects, with those involving energy balance and appetite best understood. Much of the interest in GLP-1 and its effect on energy balance stems from the obesity epidemic in Western cultures and the pursuit of drugs suitable for use in weight and Type 2 diabetes mellitus (T2DM) management. The mechanisms controlling food intake involve an interplay between gut, brain and adipose tissue, with involvement of sympathetic, parasympathetic and other neuronal

systems (Crespo et al., 2014). However, central mechanisms appear to be important in mediating the satiety effect of GLP-1, regardless of whether it is produced centrally or peripherally (Dailey & Moran, 2013). GLP 1 alters food intake through a number of central pathways including the hypothalamus, hindbrain nuclei, NTS, ventral hippocampus and nuclei in the mesolimbic reward system, (Sekar, 2017).

GLP-1 stimulates insulin release from β cells of the pancreas and also results in non-insulin dependent glucose production and utilisation by tissues (Faulkner & Martin, 1999). The strength of insulinotrophic activity is dependent on the glucose state of the animal, with basal glucose levels associated with a weak response. It is also a key regulator of glucagon secretion and function (Sandoval & D'Alessio, 2015). A 2-day infusion of GLP-1 was found to induce a trophic effect on β-cells in Zucker diabetic rats, suggesting that GLP-1 is involved in the regulation of β-cell numbers, promoting islet growth (Farilla et al., 2002).

Glucagon-like peptide 1 induces lipogenesis, decreases lipolysis and suppresses hepatic ketogenesis in sheep, in an insulin-independent manner (El-Sabagh et al., 2015; Relling, Lee, Loerch, & Reynolds, 2010). Glucagon-like peptide 1 also has anabolic effects on muscle and liver tissue and causes increased lipogenesis in adipose tissue. There is some evidence that GLP-1 enhances mineralocorticoid and glucocorticoid secretion and may affect drinking behavior, perhaps via angiotensin II (Bojanowska & Stempniak, 2003; Nussdorfer, Bahcelioglu, Neri, & Malendowicz, 2000).

Gut peptides are also reported to have a role in the physical and physiological transformation of pre-pubertal, growing animals (Fukumori et al., 2012).

It is unclear whether the central actions of GLP-1 on appetite are due to locally produced GLP-1 or peripheral, circulating GLP-1 (Sandoval & D'Alessio, 2015). Both are plausible, as the ARC is at least partly outside the blood brain barrier, at the median eminence (Clarke, 2014). This gives a possible site for GLP-1 to enter the CNS, a concept supported by abundant GLP-1 receptors (GLP-1R) in the ARC and relatively few fibres from GLP-1 producing neurons (Heppner et al., 2017). Peripherally acting GLP-1 must be able to activate brain circuits directly or indirectly to affect appetite, as has been demonstrated by various research groups and clinical observation (Dailey & Moran, 2013).

GLP-1 receptor agonists

There has been significant interest in GLP-1 from the medical community as a result of its known effects on appetite and insulin regulation. The incretin abilities of GLP-1 have gained particular interest as a therapeutic agent for type 2 diabetes mellitus (T2DM), which is characterized by a reduced or absent incretin effect (Holst, 2007). The anorexigenic nature of GLP-1 gives it a potential role in weight reduction therapies. The pharmacological interest in GLP-1 receptor agonists means much of our understanding of their function has a focus in humans, with relatively few animal-based studies besides those in rodents. The very short half-life (1-2 mins) and rapid metabolism of endogenous GLP-1 has led to the development GLP-1 receptor agonists with therapeutic potential. There are numerous GLP-1 receptor agonists approved for treatment of T2DM, namely; exenatide,

liraglutide, lixisenatide, albiglutide and dulaglutide. A seventh, taspoglutide, was associated with adverse effects including hypersensitivity reactions and so was not approved for clinical use (Madsbad, 2016). More recently, semaglutide injections have been approved for both weight management and treatment of T2DM.

Exenatide and lixisenatide are based on the exendin-4 molecule. Exendin-4 is 53% homologous to naturally occurring GLP-1 (Madsbad, 2016). A systematic search for biologically active peptides led to exendin 4 being isolated from the venom of a lizard, *Heloderma suspectum* (Eng, Kleinman, Singh, Singh, & Raufman, 1992). Exendin-4 is an equipotent, full agonist for GLP-1. It is not degraded by DPP IV but excreted by glomerular filtration, resulting in a half-life of approximately 30 minutes (Madsbad, 2016). The similar action and potency but longer half-life of exendin 4 has led to it being used in research to better understand the *in vivo* effects of GLP-1, the half-life of which is so brief it can be difficult to detect any effects (Holst, 2007). Some differences in action between exendin 4 and native GLP-1 have been found in *in vitro* studies, however the effects of the two molecules have been found to be very similar *in vivo* (Madsbad, 2016; Runge et al., 2007). There are some differences in the clinical effects of currently available GLP-1 receptor agonists. Generally, longer-acting agonists e.g. albiglutide do not have the same effect on gastric motility as short acting agonists and endogenous GLP-1 (Madsbad, 2016). An interesting point of difference is that the larger molecules, albiglutide and dulaglutide, do not appear to have the same effect on weight loss as the smaller liraglutide, a

difference potentially explained by a hindered ability of larger molecules to cross the blood brain barrier and exert a central, anorectic effect (Madsbad, 2016).

GLP-1 in ruminants

Generally, the effects of GLP-1 appear to be well preserved across mammalian species, unsurprising given the early emergence of the preproglucagon gene and GLP-1. However, much of the research to date has focused on monogastric species. The extensive microbial fermentation which takes place in the rumen and reticulum of ruminants results in differences in carbohydrate digestion and metabolism in ruminants when compared to monogastric species. Fibrous carbohydrates constitute the majority of the ruminant diet. In the reticulorumen fibrous carbohydrates are reduced to volatile fatty acids, principally acetate, propionate and butyrate. Most of the volatile fatty acids (VFAs) produced are absorbed through the ruminal epithelium and do not reach the small intestine (Pond, 1995). Ruminants use VFAs as the primary source of energy for metabolism, in contrast to monogastric animals in which the major product of carbohydrate digestion is glucose (Pond, 1995). There are significant gaps in the current knowledge of GLP-1 release and regulation in ruminants (Thanthan et al., 2012). Much of what is accepted about GLP-1 physiology in ruminants has been extrapolated from other species, although this may not always be appropriate. Perhaps unsurprisingly, whilst glucose and lipids are regulators of GLP-1 release in monogastrics, they are ineffective in the ruminant (Faulkner & Martin, 1997; Relling, Reynolds, & Loerch, 2011). Fukumori et al. (2012) found that short chain fatty acids (SCFA) stimulated GLP-1 secretion in calves using a

supraphysiological dose of SCFAs. Hatew et al., (2019) found that a ruminal infusion of VFA, particularly butyrate, stimulated GLP-1 release Although GLP-1 has been detected in the plasma of ruminants and feed-related studies confirm it has a biphasic release in response to a meal (similar to monogastrics) the mechanisms behind this release have not been given much attention to date. Indeed, to the author's knowledge, it has not yet been confirmed that intestinal L-cells are responsible for GLP-1 production and secretion as they are in monogastrics. It is also unknown at what level nutrient intake stimulates GLP-1 release e.g. a direct response of L-cells to the presence of nutrients in the intestine as opposed to an indirect response mediated by ruminal absorption or the nervous system. The pattern of release in recent studies suggests that it is likely to be secreted by an enterocyte, likely intestinal L-cells. Although it has been postulated that beta hydroxybutyrate production by ruminal epithelium may stimulate GLP-1 production this has not been supported in some studies (Hatew et al., 2019).

The insulin dependent actions of GLP-1 in ruminants are better documented, and are largely similar to the effects seen in monogastrics. In ruminants, GLP-1 has insulinotropic effects under normo- and hyper-glycaemic conditions. The insulin-independent effects of GLP-1 are less well understood, but have been shown to exist (El-Sabagh, Taniguchi, Sugino, & Obitsu, 2016). A study in sheep by El-Sabagh et al (2015) found that infusion of GLP-1 and insulin increased plasma insulin to two-fold of insulin infusion alone. That study also found that GLP-1 increased circulating NEFA, BHB and TG, a reverse of what has been reported in monogastric animals. GLP-1 also appears to have insulin-

independent effects on lipid and protein metabolism (El-Sabagh et al., 2015). Insulin plus GLP-1 infusion enhanced hepatic protein anabolism and reduced hepatic gluconeogenesis (El-Sabagh et al., 2016). Glucagon-like peptide 1 likely enhances lipolytic and ketogenic pathways in ruminants (El-Sabagh et al., 2016). Similar to monogastrics, GLP-1 reduces dry matter intake in ruminants using a hypothalamic mechanism, an effect in part mediated by long chain fatty acids (LCFA) (Fukumori, 2018; Relling et al., 2010; Relling et al., 2011). Indeed, GLP-1 secretion is likely to be responsible for the reduction in DMI seen with diets high in LCFA, particularly mono- and poly-unsaturated LCFA (Fukumori, 2018). It is uncertain whether circulating GLP-1 reduces gut motility and transit time, as it does in non-ruminants (Hatew et al., 2019; Relling et al., 2011)

An interesting observation is that GLP-1 is elevated during lactation in ruminants despite low circulating insulin and a catabolic state (Faulkner & Martin, 1997, 1998; Relling, 2007). This may be due to increased food intake, however it could also be a consequence of the proliferation of the intestine, including L cells, that occurs during pregnancy and lactation (Faulkner & Martin, 1997). There is some evidence this increase may not relate to feeding behavior but to the endocrine and metabolic adaptations of lactation (Relling, 2007). Elevated GLP-1 concentrations have been observed during lactation in cattle, sheep and goats and suggest a shift in the insulin response curve of the pancreas to GLP-1 (Faulkner & Martin, 1997, 1998; Faulkner & Martin, 1999). The effect of GLP-1 also appears to change with maturity, with different effects seen pre- and post- weaning in calves,

unsurprising given that milk-fed ruminants have an underdeveloped rumen and are essentially monogastrics (Fukumori et al., 2012).

The distribution of GLP-1 receptors in ruminants appears to be similar to monogastrics. This has not been confirmed using molecular biology but can be inferred based on the observed effects of GLP-1 in ruminant based studies. A 2004 study found that GLP-1 is a modest physiological regulator of growth hormone in sheep, suggesting that GLP-1 receptors may be present in the anterior pituitary (Kurose et al., 2004). Alternately, the effect may be indirect e.g. somatostatin release from the hypothalamus. This observation is of interest when we begin to consider the potential role of GLP-1 on the reproductive axis, which also involves hormones produced by the pituitary and hypothalamus.

GLP-1 and reproduction

Energy balance and reproduction have a strong association (*supra vide*). Our understanding of the physiology underlying this relationship is still developing. If we accept that anorexigenic pathways are generally associated with an upregulation of the HPG axis, it seems reasonable to expect that a potent appetite suppressant such as GLP-1 would be part of this mechanism. The relationship between gut peptides and reproductive function has received very little attention. However, the few studies that have considered this relationship provide encouraging evidence that GLP-1 is involved in reproductive function.

An *in vitro* study in male Wistar rats by Beak et al (1998) found that exposure of hypothalamic cells to GLP-1 increased LH releasing hormone (LHRH/GnRH) release in a dose dependent manner, whilst

intracerebroventricular injection of GLP-1 stimulated LH release when compared to saline controls. There was a significant increase in LH levels 5 minutes after injection, with no short-term effect on circulating FSH or thyrotropin. *In vivo* work on GLP-1 receptor knockout (GLP-1R -/-) mice found that GLP-1R -/- mice had small but significant decreases in testicular and seminal vesicle weight, decreased number of developing ovarian follicles and a consistent delay in puberty of 2 days in females (MacLusky et al., 2000). These changes were independent of changes in energy homeostasis, suggesting a direct effect of GLP-1 on the reproductive axis (MacLusky et al., 2000). A study in men found that a GLP-1 infusion reduced pulsatile testosterone secretion by a mechanism independent of LH release (Jeibmann, Zahedi, Simoni, Nieschlag, & Byrne, 2005). The use of a GLP-1 agonist, exenatide, was associated with resumption of normal menstrual activity in overweight women with polycystic ovarian syndrome (PCOS) (Elkind-Hirsch, Marrioneaux, Bhushan, Vernor, & Bhushan, 2008). Several women were excluded from the study after becoming pregnant during treatment. These results were confounded by weight loss, however the unique form of insulin resistance present in PCOS and the observation that improvement in menstrual bleeding frequency preceded other changes in carbohydrate metabolism may suggest direct involvement of incretins such as GLP-1 in PCOS (Elkind-Hirsch et al., 2008). Other studies have confirmed that PCOS is associated with GLP-1 dysfunction in women of a normal weight, although the nature of this relationship is uncertain (Outeirino-Iglesias et al., 2015; Vrbikova et al., 2008).

There is also neuroanatomical evidence supporting a relationship between GLP-1 and the reproductive axis. Immunohistochemistry studies have demonstrated GLP-1 immunoreactive fibres in close apposition to GnRH neurons, suggesting activation of GLP-1 producing cells in the CNS has a downstream effect on GnRH secretion (Farkas et al., 2016; Heppner et al., 2017). Kisspeptin-producing neurons in the ARC were found to co-express GLP-1R mRNA using *in-situ* hybridization (Heppner et al., 2017). Depolarisation of ARC kisspeptin neurons occurred when exposed to liraglutide, a GLP-1 receptor agonist (Heppner et al., 2017). GLP-producing fibers also come into close apposition with GnRH neurons, which may indicate that GnRH secretion can be altered by GLP-1 independently of kisspeptin cells (Farkas et al., 2016; Heppner et al., 2017). Interestingly, the density of GLP1 receptors in the ARC and median eminence is reported to be high, despite neural input from GLP-1 producing neurons being low. This is in contrast to the rest of the hypothalamus, where receptor density generally reflects the density of GLP-1 fibers (Larsen et al., 1997). This would suggest that a peripheral source of GLP-1 is more important in the ARC than centrally produced GLP-1. The observation that the median eminence sits outside the blood-brain-barrier supports this idea (Langlet et al., 2013; Larsen et al., 1997).

Conclusion

It is clear that GLP-1 has an important role in the regulation of energy via its roles in glucose metabolism and appetite suppression. These functions appear to be well conserved within mammals, including ruminants, as is the preproglucagon gene sequence. Much less is known about potential

other roles of GLP-1, particularly how it may influence reproduction, despite increasing evidence that such a relationship exists.

Investigating the relationship between GLP-1 and the reproductive axis should provide useful insight into the physiology of how some of the many known effects of nutrition and energy intake may take effect. A more thorough understanding of this relationship can inform how feeding practices may benefit reproductive outcomes, particularly in livestock species. If GLP-1 can be confirmed to upregulate reproductive activity it sets an intriguing precedent for other gut peptides and appetite-related hormones.

Our research aims to explore this relationship by observing whether GLP-1 has an effect on the hypothalamic-pituitary-gonadal axis of the ewe using infusion studies with the GLP-1 agonist, Exendin-4. This synthetic GLP-1 agonist has been chosen to explore the relationships as its greater affinity for the GLP-1 receptor and longer duration of effect increase the likelihood of a relationship being detected via changes in plasma LH concentration. We will also observe changes in plasma FSH concentration.

The ewe is a useful and well-established model in studies of reproduction, as its size and relatively docile nature are conducive to sampling and findings can typically be extrapolated to other mammalian species. Being a livestock species, findings in sheep may have more immediate relevance and applications than findings in rodent species. We also aim to confirm expression of the preproglucagon gene in the ovine intestinal tract as this has not previously been reported in the

literature. We will use real-time PCR to investigate differences in expression throughout the tract and at different stages of the oestrous cycle, although sample numbers restrict this aspect of the research to a pilot study.

We hope that this research confirms the relationship between peripherally produced GLP-1 and reproduction in the ewe and provides valuable insights into the relations between nutrition and reproduction.

Chapter 3: The effect of a GLP-1 agonist on the reproductive axis of the ewe: Preliminary study

Research Chapter 3: The effect of a GLP-1 agonist on the reproductive axis of the ewe: Preliminary study

Preface

Glucagon-like peptide 1 (GLP-1) is emerging as a potential mediator in the relationship between nutrition/metabolism and reproduction. There is a mounting body of neuro-anatomical evidence but relatively few interventional studies exploring this relationship. This preliminary study was developed to determine proof-of-concept (that peripheral administration of a GLP-1 agonist, Exendin-4, has an effect on gonadotroph secretion) in the ewe and to inform the experimental design of a larger project. Oestrus synchronization was used to form two experimental groups; Follicular phase and Luteal phase. Blood samples were collected via indwelling jugular catheters from 8 Merino ewes over an 8-hour period. Intravenous infusion of 2mg of Exendin-4 was performed over 1 hour mid-way through the sampling period. Radio-immunoassays were used to determine plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations.

This study found that Exendin-4 infusion was associated with increased concentrations of both LH and FSH. Stage of oestrous cycle had a marked effect on response, with the amplitude of response greater in ewes in the follicular phase.

Introduction

The relationship between nutrition and reproduction has long been appreciated but understanding of the physiological mechanisms underlying this important relationship is still developing. Virtually every neurotransmitter and neuropeptide in the brain has an effect on food intake and/or energy

expenditure (Clarke, 2014). The peripheral metabolites which regulate energy homeostasis are numerous, with recent research adding to the list. It is also unknown whether signaling between metabolic status and reproduction occurs centrally, at the level of the hypothalamus or pituitary, or peripherally, at the gonad. Given the complexity of energy homeostasis, multi-tiered nature of the reproductive axis and large number of hormones and metabolites involved, it seems most likely that a complex relationship exists, with multiple points of control. A better understanding of this relationship will allow more targeted manipulation of diet and feeding practices to improve reproductive efficiency in domestic animals. Research in this area is also important for human health, where the increasing incidence of obesity in Western society compromises fertility and therapeutics which may alter this relationship are increasingly common (De Bond & Smith, 2014; Madsbad, 2016).

Several hormones and neurotransmitters known for their importance in energy metabolism may have a role in reproductive function, including leptin, insulin, insulin-like growth factor and the gut peptides, including ghrelin, peptide YY and glucagon-like peptide 1 (GLP-1) (Clarke, 2014; Clarke & Arbabi, 2016b; Heppner et al., 2017; Huda et al., 2006; Larsen et al., 1997). There is a growing body of evidence that GLP-1 contributes to the relationship between metabolism and reproduction, although published research in animals is limited.

Glucagon-like peptide 1 is a 30 amino acid peptide with 50% structural homology to glucagon (Huda et al., 2006) . It is a post-translational product of the preproglucagon (*GCG*) gene (Huda et al., 2006; Sandoval & D'Alessio, 2015; Sekar, 2017) and a potent anorexigen that is secreted centrally and from

intestinal L-cells in response to eating and the presence of ingesta in the intestinal lumen (Holst, 2007; Huda et al., 2006; Paternoster & Falasca, 2018). Like most anorexigens, it is thought to upregulate reproductive function. In support of this, MacLusky et al. (2000) found that female GLP-1 receptor knock-out mice have a delayed onset in puberty. An *in-vitro* study by Beak et al. (1998) found that exposure of hypothalamic cells to GLP-1 increased GnRH release in a dose dependant manner, whilst intracerebroventricular injection of GLP-1 stimulated LH release in male rats. Immunohistochemistry studies have demonstrated GLP-1 immunoreactive fibres in close apposition to GnRH neurons, suggesting activation of GLP-1 producing cells in the CNS has a downstream effect on GnRH secretion (Farkas et al., 2016; Heppner et al., 2017). Interestingly, the density of GLP-1 receptors in the arcuate nucleus (ARC) and median eminence (ME) is high, despite relatively little neural input from GLP-1 producing neurons (Larsen et al., 1997). This would suggest a peripheral source of GLP-1 is important in the ARC. The observation that the ME sits outside the blood-brain-barrier supports this concept (Langlet et al., 2013).

Despite this encouraging evidence, there is no published research on the direct effects of peripheral GLP-1 on the reproductive axis of animals. The objective of this study was to confirm that peripheral GLP-1 has a temporal relationship with the hypothalamic-pituitary-gonadal axis. The aims of this pilot study were to i) observe the effect of administration of a GLP-1 agonist, exendin-4, on luteinising hormone (LH) and/or FSH secretion in ewes and ii) observe whether phase of oestrous cycle has an effect on response to GLP-1 agonist.

Materials and Methods

Animal care and ethics statement

This study protocol was approved by the Charles Sturt University Animal Care and Ethics Committee on the 10th May 2018, approval number A18029.

Animals

Twelve five-year-old Merino ewes were recruited from the Charles Sturt University teaching and research flock. The study was performed in January, 2019, during the Australian summer. All ewes were in a mean body condition score of 2.5/5 (Jefferies 1961) at the time of the study and weighed 49-59 kg (mean 52.95, median 53). Ewes were assigned to either the Luteal phase or Follicular phase group and their oestrous cycle synchronised accordingly. There were 6 ewes in each group. All ewes were returned to the teaching flock at the conclusion of the study.

Oestrus synchronisation

An intravaginal progesterone releasing device (EAZI-BREED CIDR® Sheep, Zoetis Australia, Rhodes, NSW Australia) was aseptically inserted into the vagina 21 days before the day of sampling in the Luteal group. The CIDRs were removed 14 days later and 250 IU of equine chorionic gonadotrophin (Novormon, Zoetis Australia, Rhodes, NSW Australia) was given intramuscularly.

The synchronisation protocol was the same in the Follicular group but delayed by 5 days. The aim of the synchronisation program was for all ewes in the Luteal group to have ovulated recently and have an active corpus luteum (CL) and ewes in the Follicular group to be in the follicular phase with no active CL s on the day of sampling.

Housing

All ewes were housed in individual pens in the Charles Sturt University Animal House. While housed, all ewes were given 1.4kg of a lucerne chaff mix (Corowa Chaff Mill, Corowa NSW) twice daily except the day of experiment when they were fed once, in the evening. Water was provided *ad libitum* throughout the housing and experimental period. Ewes were acclimatised for 5 days prior to sampling.

Jugular catheterisation

Indwelling jugular catheters were placed the day prior to infusion and sampling.

The infusion line was placed as follows: The ewe was placed in a sitting position with the head and neck placed over the right leg of the person performing catheterisation to expose the left side of the neck. The wool on the left side of the neck was clipped using a #40 clipper blade. The skin was aseptically prepared with chlorhexidine and alcohol. The jugular vein was raised and lignocaine 20mg/mL (Troy Laboratories Pty Ltd, Glenderring NSW) was applied topically over the anticipated site of catheterisation. The vein was raised and a 12G hypodermic needle was inserted through the skin incision into the jugular vein. A 60cm length of 0.86mm medical grade polyethylene tubing (Sterihealth Laboratory Products Pty Ltd, Dandenong Sth, Vic, Australia) primed with heparinised saline was inserted through the needle into the vein and advanced 10cm. The 12G needle was then removed over the tubing. A blunted, 21G needle with injection port was placed into the other end of the tubing. The tubing was secured with 2/0 non-absorbable pseudomonofilament polyamide suture (“Supramid”, B. Braun, Germany) using a “Chinese finger-trap” suture pattern in two places then

under a light bandage placed around the neck. Both the infusion line and sampling line were checked for patency before the ewes were returned to their pens and the following day before sampling began

The sampling line was placed as follows: Wool and skin on the right side of the neck was clipped and prepared as for the infusion line. A #21 scalpel blade was used to make a small skin incision over the left jugular vein. A 12G catheter (Dwellcath, Tuta Laboratories Australia Pty Ltd, Lane Cove NSW Australia) was inserted through the skin incision into the jugular vein. The stylet was removed and the catheter hub secured to the skin using superglue and adhesive bandage (“Elastoplast” Beirsdorf Australia Ltd, North Ryde NSW Australia). A 75cm extension set (Global Veterinary Products, Queensland Australia) primed with heparinised saline was then attached to the catheter hub and secured to wool using rubber bands. A three way stop cock was attached to the other end of the giving set and secured under a light neck bandage as above.

a)

b)

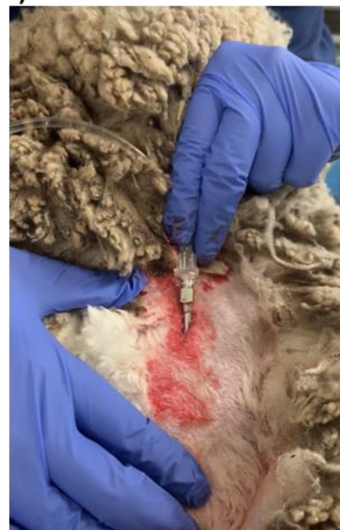

Figure 3.1 Ewes in individual pens during acclimatisation period (a) and jugular catheter and sampling line *in situ* for preliminary study on the effect of Exendin-4 infusion on LH and FSH secretion in the ewe.

A patent catheter was not achieved in two ewes from the follicular phase group. These ewes were excluded from sampling. Two ewes were subsequently excluded from the luteal phase group to avoid sample size bias.

Table 3.1 Live weight, stage of cycle, serum progesterone concentration and sampling notes for ewes recruited into the preliminary study

Ewe #	Weight	Stage of cycle	P4 (nmol/L)	Notes
A11	53.5	luteal	3.4	
A21	53	luteal	5.1	
A31	54.5	luteal	4.2	
B11	53	follicular	<.6	
B21	49.5			Excluded
B31	52	follicular	<0.6	
C11	52			Excluded - only one jugular catheterised
C21	55	follicular	<0.6	
C31	59			Excluded - difficult to catheterise
D11	54	follicular	<0.6	
D21	49			Excluded
D31	51	luteal	3.8	

Serum progesterone sampling and assay

A single 3-5mL blood sample was collected from the sampling line into a plain (red top) vacutainer immediately prior to the sampling period, for progesterone assay. Sampling technique was as

described below. Samples were analysed the same morning at the Charles Sturt University Veterinary Diagnostic laboratory using solid phase, competitive immunoassays (IMMULITE 1000, Siemens Healthcare, Hawthorn East, VIC, Australia).

Sampling procedure

Blood samples were collected every 10 minutes for a period of 8 hours. Sampling was performed using a three-way stopcock on the sampling line. Before each sample was taken, 5mL of fluid (admixture of heparanised saline and blood) was withdrawn from the sampling line and discarded. A further 5mL of blood was withdrawn and immediately placed in a lithium heparin vacutainer tube. The sampling line was then flushed with 5-10mL of heparinised saline and replaced within the light bandage. Sampling was performed by two samplers and two assistants at each interval. Sampling of all 8 ewes took approximately 5 minutes and was done in the same sequence for each interval. Packed cell volume was visually assessed throughout the sampling period and remained within normal parameters (27-45%) (Edmondson, Roberts, Baird, Bychawski, & Pugh, 2012).

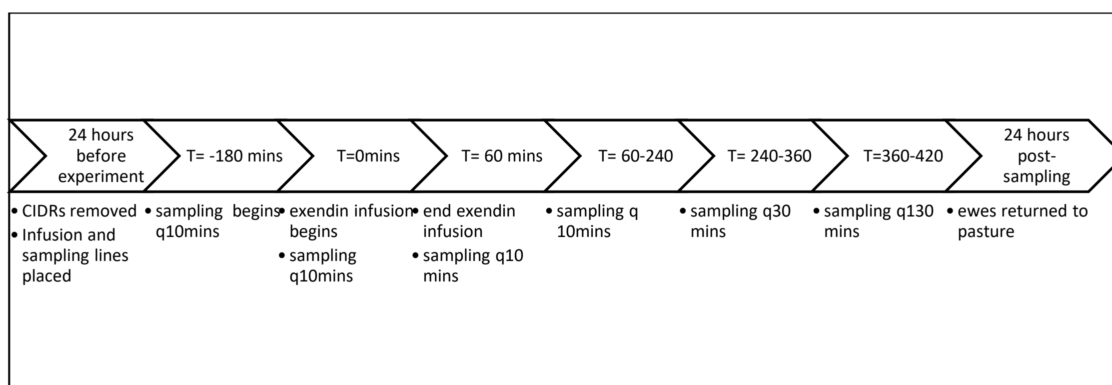

Figure 3.2 Timeline of events in the preliminary experiment on the effect of Exendin-4 infusion on gonadotroph secretion in the ewe

Samples underwent centrifugation at 4000 rpm for 10 minutes. Plasma was aspirated from the top of the sample into a separate tube and stored at -20°C. Samples were maintained at-20°C until they were shipped frozen for analysis of LH concentrations.

Exendin-4 (GLP-1 agonist) infusion

Exendin-4 (ab120214; Abcam) was stored at -20°C until the day of experimentation. A small amount of sterile water for injection was added to each 1mg vial of exendin, mixed to allow the exendin to dissolve and then aspirated. The infusion solution was prepared by mixing 2mg of Exendin-4 with 4mL of sterile water for injection in a 5mL syringe. Each syringe was then attached to a syringe pump (Graseby MS16A syringe driver, Smiths Medical Minneapolis, Minn. U.S.A), placed within a pack and fastened to the dorsum of each ewe with adhesive bandage. The infusion line was then attached to the syringe with a 24 gauge ½ inch needle whose blunted tip was inserted within the infusion line. The infusion line was primed with 0.3mL of the Exendin-4 solution. The infusion was started immediately after the 180 min sample and ran for one hour, at a rate of 4mL/hr. Ten-minute sampling continued throughout this period as described above. The infusion pumps were visually assessed at each sampling point to ensure the infusion was progressing appropriately. Once the entire infusion had been administered, a 4mL syringe containing water for injection was attached for a further 20

minutes to flush the infusion line of any remaining Exendin-4. All pumps were stopped and removed between sampling periods, the infusion line capped with an infusion port and replaced within the protective bandage.

Post-infusion monitoring

All ewes were fed at the end of the sampling period and all indwelling catheters removed. Ewes remained in their individual pens for 24 hours, with visual assessment occurring twice daily. Appetite, demeanour and rectal temperature remained within normal limits. The ewes were returned to a small paddock for a period of two weeks and visually checked daily. They were then returned to their usual flock.

Radioimmunoassays for LH analysis

Radioimmunoassay of plasma samples was used to measure LH concentrations using NIADDK anti-ovine (o) LH-1 as antiserum and NIH-0LH-S18 as a standard. Iodinated ovine LH (1251-NIDDK-AFD-9598B) was used as a tracer. Assay sensitivity was 0.069ng/mL and intra-assay CV less than 10% between 0.337-11.075ng/mL

Radioimmunoassays for FSH analysis

Ovine plasma FSH concentrations were measured in a homologous double antibody radioimmunoassay (RIA) performed using FSH antiserum raised in rabbit (NIDDK-anti-oFSH-1). Ovine FSH (NHPP-oFSH-SIAFP-RP-2) was used as standard and highly purified ovine FSH (NHPP-USDA-oFSH-19-SIAP) was used for iodination. Assay sensitivity was 0.064ng/mL and intra-assay CV less than 10% between 0.377-22.598 ng/mL.

Statistical Analysis

After using Microsoft Excel (Microsoft Corporation, 2018) for data entry, R (R Core Team 20) was used for statistical analysis.

For the purpose of analysis, serum progesterone concentrations <3nmol/L were considered consistent with the follicular phase, values >3nmol/L with the luteal phase.

Following exploratory data analysis, a multiple linear regression model was fitted to the data using LH concentration as the response variable. Logarithmic transformation of the data was performed after a diagnostic check of the model, to achieve homogeneity of variance. Paired t-tests were used to determine when LH values rose above baseline. LH pulse analysis was performed using an accepted method (Arbabi et al., 2021). An LH pulse was defined as having a value higher than three times the assay S.D. of the preceding LH value. The inter-pulse interval (IPI) was calculated as the time between the start of two adjacent pulses. A mixed effect regression model was fitted to the data for inferential analysis. A diagnostic check of the assumption conditions for the model suggested a logarithmic transformation was warranted. The “emmeans” function of the “emmeans” package in R was used for pairwise comparison (Lenth, 2023).

The FSH assay sensitivity value (0.064ng/mL) was used for values below detectable levels. This occurred in one ewe from the follicular phase group during the pre-infusion phase. After exploratory data analysis a regression model was fitted to the data. An Akaike information criterion (AIC) (Crawley, 2013) check suggested that a linear mixed model without interaction terms or logarithmic

transformation was most suitable. The “emmeans” function of the “emmeans” package in R was used for pairwise comparison (Lenth, 2023).

Results

Progesterone

All ewes in the follicular phase group had serum progesterone concentrations below the assay sensitivity (0.6nmol/L). Ewes in the luteal phase group had serum progesterone concentrations ranging from 3.4 to 5.1 nmol/L (Table 3.1).

Plasma concentrations of luteinising hormone

Plasma LH concentrations were below the assay sensitivity (0.069ng/mL) for all ewes in the luteal phase group prior to the infusion period. Infusion of Exendin-4 increased LH secretion in both the luteal and follicular phase groups ($p<0.001$) (Fig 3.2). The effect continued in the post-infusion period ($p<0.001$). Plasma LH concentrations were higher in the follicular phase group than the luteal phase group during and after the infusion period ($p< 0.01$ and $p<0.001$ respectively) (Fig. 3.4).

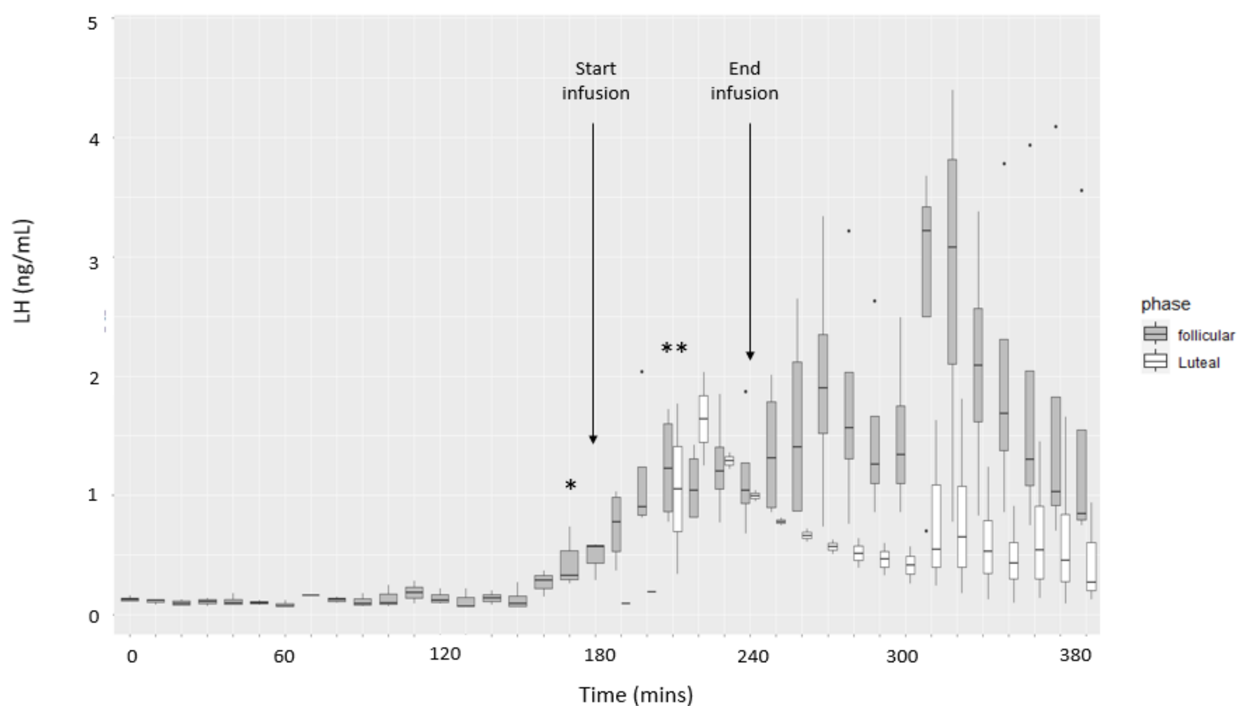

Figure 3.3 Plasma LH concentrations in the ewes of the preliminary study, treated with 2mg of Exendin-4 in either the follicular (n=4) or luteal phase (n=4) of the oestrous cycle. The data are presented as box plots, where the lower and upper margins of each box represent the 25th and 75th quartiles respectively. The solid lines within each box represent the median values. The vertical lines represent the minimum and maximum values of the data and dots represent outliers. The two solid lines mid-graph represent median values in the luteal phase group for which there were too few values to construct a box. ** $p<0.01$ compared to pre-infusion mean

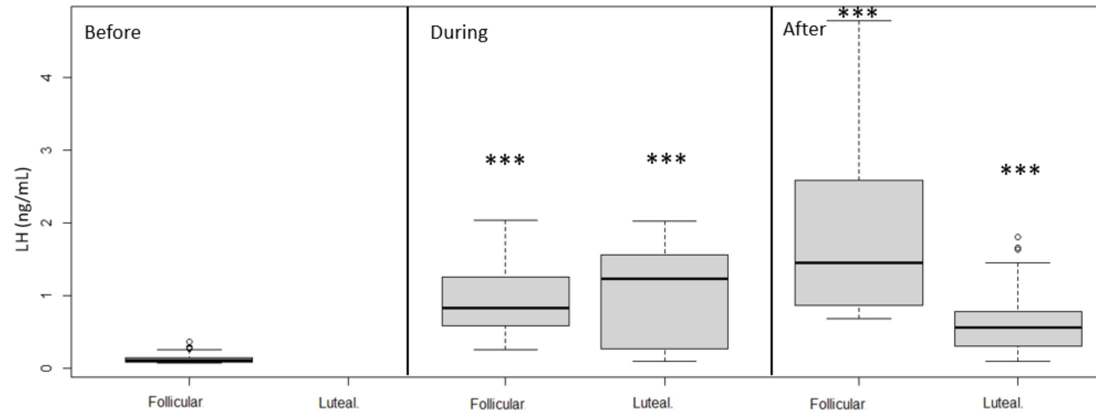

Figure 3.4 Boxplot of LH response to treatment of ewes in either the follicular or luteal phase of the oestrous cycle with 2mg of Exendin-4 pre-infusion (t=-0-180mins), during infusion (t=180-240mins) and post-infusion (t=240-420mins) of Exendin-4. *** p ≤0.001 vs pre-infusion

Two ewes in the luteal phase group had no LH pulses during the observation period and were excluded from pulse analysis. Exendin-4 infusion increased LH pulse amplitude during and after the infusion period (p<0.01 and p<0.001 respectively) (Fig 3.5). Pulse amplitude was greater in the follicular phase than the luteal phase at all stages of the experiment (p<0.001). Inter-pulse interval was shorter in the follicular phase than the luteal phase at all stages of the experiment, however treatment had no effect.

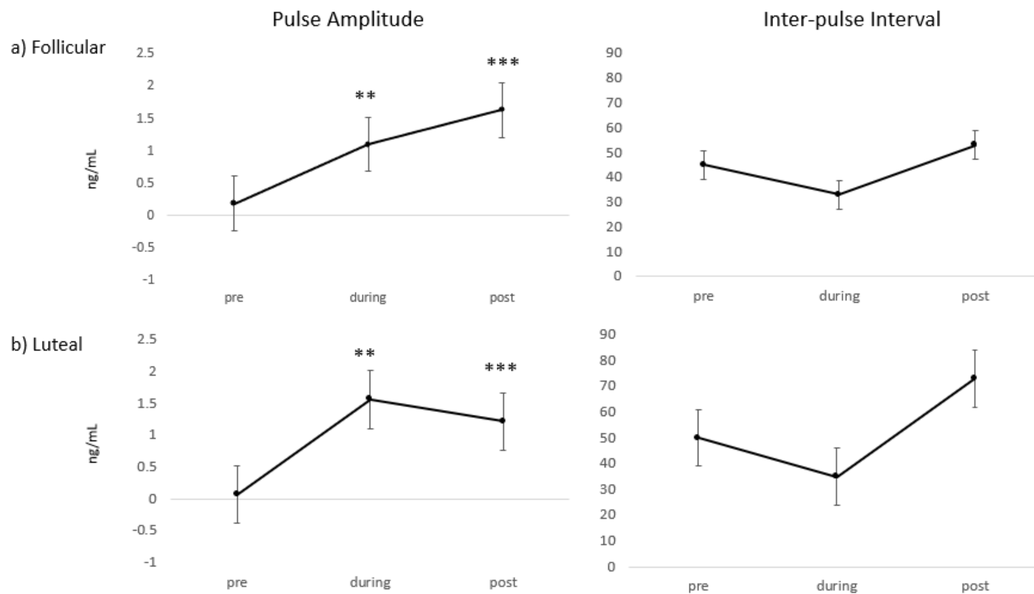

Figure 3.5 Mean ± SEM plots of LH pulse amplitude in and LH inter-pulse interval pre- (t=-0-180mins), during (t=180-240mins) and post- (t=240-420mins) infusion of Exendin-4 in the a) Follicular group, b) Luteal group. *** p <0.001 vs pre-infusion ** p<0.01 vs pre-infusion

Plasma concentrations of follicle stimulating hormone

Exendin-4 infusion increased FSH secretion in both the follicular and luteal phase (p<0.0001). This effect continued after infusion in both groups (p<0.0001) (Fig 3.6). Concentrations of FSH were higher in the luteal group than the follicular group at all stages of the experiment (p<0.1) (Fig 3.7).

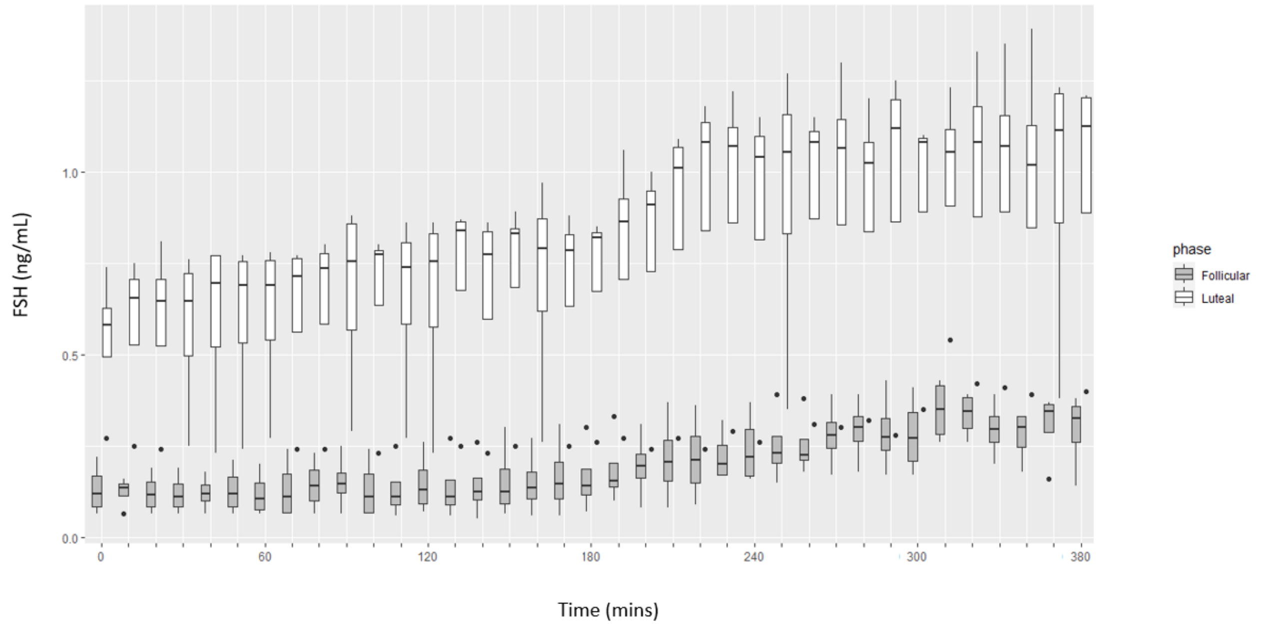

Figure 3. 6 Boxplot of plasma FSH concentration over time in the ewes of the preliminary study, treated with 2mg of Exendin-4 in either the follicular (n=4) or luteal phase (n=4) of the oestrous cycle.

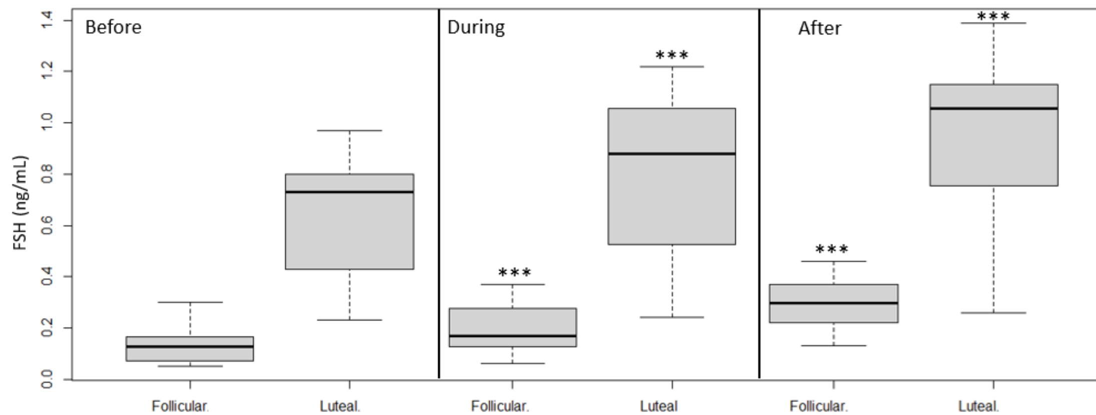

Figure 3.7 Boxplot of FSH response to treatment of ewes in either the follicular or luteal phase of the oestrous cycle with 2mg of Exendin-4 . pre- (t=-0-180mins), during (t=180-240mins) and post- (t=240-420mins) infusion. *** p<0.001 vs pre-infusion

Discussion

This study confirms that systemic administration of GLP-1 can influence the hypothalamic-pituitary-gonadal axis in sheep, providing the proof of concept to validate a more extensive study. This finding is unsurprising, given the mounting neuroanatomical and medical evidence that there is a relationship between GLP-1 and reproductive function (Arbabi et al., 2021; Elkind-Hirsch et al., 2008; Han, 2019; Heppner et al., 2017; Larsen et al., 1997; Salamun, Jensterle, Janez, & Vrtacnik Bokal, 2018). Previous study of the direct effect of GLP-1 on the reproductive axis has been by microinjection into the median eminence (Arbabi et al., 2021) or *ex vivo* (Farkas et al., 2016). The ME contains GnRH neurons and has a dense population of GLP-1 receptors, although there does not appear to be co-expression of GLP-1 receptors on GnRH-producing neurons (Arbabi et al., 2021; Larsen et al., 1997). Microinjection of GLP-1 into the ME of the ewe resulted in increased LH secretion, whilst exposure of brain slices from male mice demonstrated a direct, excitatory effect of Exendin-4 on GnRH neurons (Farkas et al., 2016). It has thus previously been uncertain whether a peripheral source of GLP-1, such as that produced by intestinal L-cells, may be able to increase gonadotrophin secretion *in vivo*.

The use of intact, cycling ewes synchronised to be at different stages of the oestrous cycle in this study demonstrated that the effect of GLP-1 varies depending on hormonal conditions. The response of LH secretion to Exendin-4 infusion was more pronounced and appeared more rapid in ewes in the follicular phase, when plasma progesterone concentrations are low and oestrogen concentrations high. This makes biological sense, given that progesterone exerts negative feedback on kisspeptin,

suppressing GnRH release from the hypothalamus (Clarke, 2018). The ability of Exendin-4 to overcome this suppression to an extent as demonstrated by the increase in LH secretion to detectable levels in the Luteal phase group is further evidence of the role of GLP-1 in control of reproduction. The difference in response seen could be due to i) increased production of GLP-1 during oestrus ii) increased sensitivity of the reproductive axis to GLP-1 during oestrus or iii) a combination thereof. A study of the rat hypothalamus found that GLP-1R expression varies during the oestrous cycle, although in that study receptor expression was highest during dioestrus and lowest during pro-oestrus and oestrus; results that seem at odds with our study (Outeirino-Iglesias et al., 2015). Johnson, Saffey, and Taylor (2017) found that GLP-1 production and plasma GLP-1 concentrations were greatest during pro-oestrus in the rat. This would suggest that both GLP-1 production and receptor function contribute to the differential response of the pituitary to GLP-1. Further research is needed to determine whether these effects are seen in non-rodent species, including confirming of preproglucagon (*GCG*) gene and GLP-1 receptor expression across the oestrous cycle at different sites of the gastrointestinal tract and hypothalamic-pituitary-gonadal axis to determine how this effect might be mediated. It is unclear whether the difference in response to GLP-1 is due to changes in oestrogen, progesterone, or other hormones such as inhibin, activin, RFRP3 or follistatin and whether the effects are direct or indirect. Our observation that response to a GLP-1 agonist differs between the follicular and luteal phase may explain some of the variation in findings between studies of GLP-1

on the reproductive axis, such as those in male animals, ovariectomised animals and those under the influence of progesterone eg during pregnancy.

The importance of reproductive status when studying the reproductive axis is highlighted by the other observations in this study, most of which reflect GnRH conditions at the time of sampling. GnRH secretion results in the downstream secretion of both LH and FSH. However, the relative secretion of gonadotroph changes relative to GnRH pulsatility. In most species, a slow-frequency GnRH pulse, as occurs during the luteal phase under the influence of progesterone, will result in upregulation of the FSH β subunit gene and increased baseline secretion of FSH (Pak & Chung, 2011). The rate of clearance of FSH is also thought to be decreased during the luteal phase. The less glycosylated or acidic isoforms of FSH produced in the absence of progesterone (ie during the follicular phase) have a shorter half-life than those produced during the luteal phase (Ulloa-Aguirre, Maldonado, Damián-Matsumura, & Timossi, 2001). This could be seen in the present study, with plasma FSH concentrations higher in ewes in the luteal phase than those in the follicular phase at all stages of the experiment. In ewes, increased FSH secretion is associated with follicular wave emergence, which typically occurs on days 0-1, 5-6 and 10-13 of the oestrous cycle, during the luteal phase (Bartlewski et al., 1999). The luteal phase ewes in the present study were at day 5-6 of the oestrous cycle which coincides with one of the above stages of increased endogenous secretion of FSH. Notably, changes in FSH secretion do not require pulsatile GnRH and are secreted in a passive manner in direct relation to the releasable pool of FSH in the pituitary. This, together with the relatively longer plasma half-life,

means changes in FSH concentrations due to altered GnRH secretion are often slower than changes in LH (Clarke, 2014; Goodman, 2015). This occurred in the present study and underscores the value in measuring LH secretion as a surrogate for GnRH secretion.

Conversely, plasma LH concentration was higher in ewes in the follicular phase than those in the luteal phase at all points of the experiment, including before infusion. Again, this difference between groups makes sense if we consider the underlying biology. During the pre-ovulatory period, GnRH pulses increase in frequency, promoting LH β subunit gene expression and resulting to increased LH pulse frequency and increased LH secretion from the pituitary, culminating with the pre-ovulatory LH surge (Pak & Chung, 2011). There is an inverse relationship between LH pulse frequency and pulse amplitude, whereby amplitude decreases as pulse frequency increases (Clarke, 2014). Interestingly, in this study infusion of Exendin-4 appeared to be able to overcome this relationship to a degree, as pulse amplitude in the follicular phase group increased following Exendin-4 infusion. This was despite no change in inter-pulse interval, suggesting the GnRH pulse generator was likely already at its maximal intrinsic rate, as would be expected in the pre-ovulatory period.

One ewe (C2) from the follicular phase group had a sharp rise in LH concentration near the end of the observation period. Whilst this may be a result of Exendin-4 infusion, it is possible that we captured the beginning of the pre-ovulatory LH surge in this ewe, albeit earlier than expected based on our synchronisation protocol. It would be worthwhile extending the observation period following Exendin-4 infusion to examine whether there is any effect on timing of the pre-ovulatory LH surge in

future studies. Further study should also consider whether there is a dose effect and whether GLP-1 exerts the same effect as the agonist Exendin-4. This preliminary study suggests that the follicular phase is associated with the most pronounced response and is most suitable for further study in the ewe.

Conclusion

Intravenous infusion of the GLP-1 agonist Exendin-4 increases secretion of LH and FSH in the ewe, a finding which suggests that the site of action is likely to be upstream at the hypothalamus, at the level of GnRH neurons and/or kisspeptin-producing neurons. An effect could be seen during both the luteal and the follicular phase but was more pronounced during the follicular phase. We were able to confirm proof-of-concept and the validity of our experimental procedure for further research into the link between GLP-1 and reproduction. A more extensive study can be designed based on this preliminary study. Our findings suggest the follicular phase is most appropriate to observe changes in LH secretion as a result of exposure to a GLP-1 agonist. A 2mg dose of Exendin-4 is effective, however further study should include a control group and a lower (closer to physiological) dose to further understand the strength of the relationship between GLP-1 and the reproductive axis. It would also be worthwhile investigating whether there is a difference in effect between GLP-1 and the GLP-1 agonist, Exendin-4.

Chapter 4: The GLP-1 agonist, Exendin-4, stimulates LH secretion in female sheep.

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The GLP-1 agonist, Exendin-4, stimulates LH secretion in female sheep.

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Short Title: Exendin 4 stimulates LH secretion in ewes

Keywords: Preproglucagon, Exendin-4, sheep, luteinising hormone

Abstract

Our previous studies showed that microinjection into the median eminence of the sheep of glucagon-like peptide- 1 (GLP-1) or its receptor agonist Exendin-4 stimulates luteinizing hormone (LH) secretion but it is unknown whether the same effect may be obtained by systemic administration of the same. The present study measured the response in terms of plasma LH concentrations, to intravenous (iv) infusion of Exendin-4. A preliminary study showed that infusion of 2mg Exendin-4 into ewes produced a greater LH response in the follicular phase of the oestrous cycle than the luteal phase. Accordingly, the main study monitored plasma LH levels in response to either 0.5mg or 2mg Exendin-4 or vehicle (normal saline) delivered by jugular infusion for 1h in the follicular phase of the oestrous cycle. Blood samples were collected at 10 min intervals before, during and after infusion. Both doses of Exendin-4 increased mean plasma LH concentrations and increased LH peripheral pulse amplitude. There was no effect on inter-pulse interval or timing of the preovulatory LH surge. These doses of Exendin-4 did not alter plasma insulin or glucose concentrations. Quantitative PCR of the gastrointestinal tract samples from a population of ewes confirmed expression of the preproglucagon gene (*GCG*). Expression increased aborally and was greatest in the rectum. It is concluded that endogenous GLP-1, most likely derived from the hind-gut may act systemically to stimulate LH secretion. The present data suggest that this effect may be obtained with levels of agonist that are lower than those functioning as an incretin.

Introduction

The level of nutrition and body composition has a significant effect on reproductive function (Clarke & Arbabi, 2016). In ruminants, as in other species, energy and nutrient restriction reduces secretion of gonadotrophin releasing hormone (GnRH), luteinising hormone (LH) and follicle stimulating hormone (FSH), may delay puberty, delay the return to cyclicity following parturition or lactation. This is associated with reduced conception rates, reduced ovulation rates/ovulation failure and poor quality oocytes (De Bond & Smith, 2014; Dupont et al. 2014; Robinson, 1996; Robinson et al. 2006). Conversely, beef cows in good condition at calving and receiving a high intake diet post-calving have a shortened anoestrous period (Wright et al. 1992), whilst nutritional “flushing” of ewes with high quality feed before mating increases ovulation rate and litter size (Robinson et al. 2006).

These effects of nutrition on reproduction are, at least in part, relayed to GnRH neurons and/or gonadotrophs by a range of hormones and neuropeptides. In general, neuropeptides that stimulate food intake have an inhibitory effect on the gonadotrophic axis, whereas those which reduce food intake have a stimulatory effect on the same (Clarke, 2014). For example, the orexigenic peptide, neuropeptide Y (NPY) reduces pulsatile LH secretion (Barker-Gibb et al. 1995), whereas melanocortins, which are anorexigenic, stimulate pulsatile LH secretion in OVX ewes (Backholer et al. 2010). The function of these neuropeptides is regulated by several appetite-regulating hormones. Thus, leptin which is a fat-derived hormone, reduces appetite but has a positive effect on the gonadotrophic axis, restoring pulsatile LH secretion in undernourished OVX ewes (Henry et al. 2004).

Conversely, the appetite stimulant ghrelin, which originates predominantly from the abomasum and small intestine in sheep, (Grouselle et al. 2008; Hayashida et al. 2001), reduces pulsatile LH secretion in OVX ewes (Iqbal et al. 2006).

Another circulating appetite regulating peptide is glucagon-like peptide 1 (GLP-1) encoded by the preproglucagon gene (*GCG*) (Huda et al. 2006; Sandoval & D'Alessio 2015; Sekar 2017). It is an anorexigen, secreted from intestinal L-cells in response to eating, and the presence of ingesta in the intestinal lumen (Paternoster & Falasca 2018) as well as being found in neurons of the nucleus of the solitary tract in the hindbrain (Jin et al. 1988; Paternoster & Falasca 2018), which project to rostral centres in the brain. With the glucose-dependent insulinotropic polypeptide (GIP), GLP-1 is an important incretin (Baggio & Drucker 2007) and GLP-1 agonists are widely used in management of type 2 diabetes mellitus (Wang et al. 2022).

There are various indications that GLP-1 may regulate the gonadotrophic axis, possibly at the level of the GnRH neurons. Brainstem derived neural elements directly contact GnRH neurons in male mice (Vastagh et al. 2021) and GLP-1 receptors are found in GnRH neurons (Farkas et al. 2018). Accordingly, GLP-1 can increase firing of GnRH cells (Vastagh et al. 2021). Kisspeptin is a neuropeptide that stimulates GnRH secretion (Ezzat et al. 2015) and central GLP-1 neurons also project to kisspeptin cells in mice (Heppner et al. 2017). The same authors demonstrated increased firing of kisspeptin cells with application of Liraglutide, a GLP-1 receptor agonist. Others have reported that GLP-1 increases kisspeptin gene expression in fetal rat brain cultures (Beak et al. 1998; Oride 2017) and GLP-1

stimulated GnRH secretion from isolated male rat hypothalami. Intracerebroventricular injection stimulated LH release in rats (Beak et al. 1998), which may be due to an effect at the level of the median eminence (ME). Another study in rats (Outeirino-Iglesias et al. 2015) presented confusing data, showing that GLP-1 magnified the pre-ovulatory LH surge whereas Exendin-4 (GLP-receptor agonist) paradoxically reduced the surge. In the same study, both agonists had no effect on basal LH secretion in dioestrous rats. GLP-1 had no effect on LH secretion in men (Jeibmann et al. 2005).

It has been reported that microinjection of either GLP-1 or Exendin-4 into the ME stimulated LH secretion in OVX ewes, strongly suggesting action at the level of GnRH terminals (Arbabi et al. 2021). Given that the ME is outside the blood brain barrier, it is possible that circulating GLP-1 can stimulate the gonadotrophic axis through action at this level. On the other hand, GLP-1 can cross the blood-brain barrier (Fu et al. 2020) raising the question as to whether circulating peptide originating from the hind gut acts on kisspeptin and/or GnRH cells. Accordingly, the objective of the present study was to determine the effect of a systemically administered GLP-1 agonist (Exendin-4) on LH secretion in ewes. We also confirmed that GLP-1 is produced predominantly in the hind gut of sheep.

Materials and Methods

All *in vivo* procedures were conducted in accordance with the Australian Prevention of Cruelty to Animals Act 1986 and the Australian code of practice for the care and use of animals for scientific purposes. All studies were approved by the Charles Sturt University Animal Care and Ethics Committee, approval numbers A18029, A19005 and A19385. Ewes of sound health were sourced

from the Charles Sturt University teaching flock. The ewes were maintained under climate-controlled conditions with diurnal lighting and provided with food (1.4kg commercial lucerne chaff mix) twice daily and water *ad libitum*. Ewes were acclimatised to being confined in pens for one week prior to the study.

Experimental Procedure for the Preliminary Study

A preliminary study was performed on 12 five year old Merino ewes with a mean body weight of 53kg (BCS 2.5/5), to ascertain whether peripheral infusion of the GLP-1 agonist (ab120214; Abcam, Cambridge, UK) stimulates LH secretion during the sheep's natural breeding season. Ewes were assigned to either Follicular phase or Luteal phase groups prior to oestrus synchronisation. In the Luteal phase group, oestrus was synchronised by aseptically inserting an intravaginal progesterone releasing device impregnated with 0.33g progesterone (EAZI-BREED CIDR, Zoetis Australia, Rhodes, NSW Australia) 21 days prior to the day of sampling. The CIDRs were removed 14 days later and 250IU of equine chorionic gonadotrophin (eCG) (Novormon, Zoetis Australia, Rhodes, NSW Australia) was administered i.m. The synchronisation protocol was the same for the Follicular phase group, but delayed by 5 days, such that CIDR removal and injection of eCG occurred 24h before sampling began. The aim of this synchronisation program was for all ewes in the Luteal Phase group to have ovulated recently, with an active corpus luteum (CL) and all ewes in the Follicular Phase group to be in the follicular phase on the day of sampling. Stage of cycle was confirmed with progesterone assays on the morning of sampling.

A 12G catheter (Dwellcath, Tuta Laboratories Australia Pty Ltd, Lane Cove NSW Australia) was inserted into the right external jugular on the day prior to sampling. A 75cm extension set (Global Veterinary Products, Queensland Australia) primed with heparinised saline (75 units of heparin/ml) and a three-way stopcock attached to allow sampling. A 12G needle was used to insert 0.86mm medical grade polyethylene tubing (Sterihealth Laboratory Products Pty Ltd, Dandenong Sth, Vic, Australia) primed with heparinised saline 15cm into the left jugular vein, which was kept patent with heparinised saline (*vide supra*); this was used for infusions (*vide infra*). A light neck bandage was used to protect both catheters. Patency of the catheter was not maintained in 2 ewes from the Follicular phase group and these animals were excluded from the experiment and 2 ewes subsequently excluded from the luteal phase group to avoid sample size bias.

A single 5mL blood sample was collected prior to the sampling period for progesterone assay. Then, blood samples (5ml) were collected every 10 min for 7h and two further samples were taken at 30min intervals, for a total sampling time of 8h. Samples were immediately placed in lithium heparin vacutainer tubes. Packed cell volume was visually assessed throughout the sampling period and remained within normal parameters. The samples were centrifuged and plasma obtained by aspiration and frozen at -20C.

After 3h of sampling the GLP-1 agonist, 2mg of Exendin-4, was infused for 60 mins (i.v.) at a rate of 2mL/h using syringe pumps (Graseby MS16A syringe driver, Smiths Medical Minneapolis, Minn. U.S.A) attached to the backs of the sheep.

Experimental Procedure for the Main Study

The experiment was performed in two replicates, in the autumn/summer of consecutive years. The ewes in the first cohort were 5 years old with a mean bodyweight of 52kg (mean BCS 2.5/5). The ewes in the second cohort were 8 years old with a mean bodyweight of 55kg (mean BCS 2.5/5). Fifteen and 16 ewes respectively were recruited into each replicate and underwent oestrus synchronisation, but only 12 ewes from each cohort underwent confinement, treatment and sampling. Acclimatisation and husbandry occurred were as described for the preliminary study. Following the results from the preliminary study, all ewes underwent oestrous synchronisation and were treated/sampled in the follicular phase of the cycle. For synchronisation, CIDRs were inserted 13-16 days before the first day of sampling and 125μg cloprostenol (Estromil, Troy Laboratories Pty Ltd) was administered i.m 3-6 days before the first day of sampling, to cause lysis of any remaining corpora lutea. The CIDRs were removed 24 hours prior to sampling, causing onset of a follicular phase of ovarian function (confirmed by measurement of progesterone in plasma).

Indwelling jugular catheters were inserted as described above. Ewes were randomly assigned to 3 groups; saline (vehicle), 0.5mg or 2mg Exendin-4, using an online random number generator (n=4 ewes/group). The sampling procedure was as described for the preliminary experiment. Sampling occurred every 10 minutes from t= -180mins to t=240mins, relative to the start of infusions. Then, 3 consecutive samples were taken at 30min intervals, followed by sampling at 2h intervals until t=2260mins, to monitor the occurrence of pre-ovulatory LH surges. Plasma insulin and glucose assays

were performed from samples collected at the midpoint of each experimental period i.e., before, during and after infusion.

Food was withheld for 12 hours prior to the beginning of the sampling period. Following the infusion, each ewe was given an evening and a morning feed throughout the experiment. Behaviour and feed refusals were monitored and recorded.

Plasma/Serum Assays

Progesterone levels in serum were measured in a solid phase, competitive immunoassay using enzyme-labelled chemiluminescent technology (IMMULITE 1000, Siemens Healthcare, Hawthorn East, VIC, Australia).

Plasma LH levels were measured by radioimmunoassays (RIA) (Lee et al 1976) using NIADDK anti-ovine LH-1 as the primary antiserum and NIH-0LH-S18 as a standard. Iodinated ovine LH (^{125}I-NIDDK-AFD-9598B) was used as a tracer. Assay sensitivity was 0.1ng/mL and intra-assay coefficient of variance (CV) less than 10% between 0.337-11.075ng/mL in the preliminary study. Assay sensitivity was 0.06-0.84ng/mL and intra-assay CV was less than 10% between 0.407-10.17ng/mL and inter-assay CV 5.7% at 6.23ng/mL and 7.9% at 12.29ng/mL.

Plasma glucose concentrations were measured using an Accu-Chek® Guide Me blood glucose monitor and test strips (Roche Diabetes Care, Mannheim, Germany).

Plasma insulin concentrations were measured in a homologous double antibody RIA. The RIA used purified insulin antiserum raised in guinea pig (Antibodies Australia, Melbourne, VIC, Australia) and

purified bovine insulin for iodination and standard (Sigma-Aldrich Pty Ltd, cat#I5500 Castle Hill, NSW, Australia). Assay sensitivity was 0.54 μIU/mL and intra-assay CV 6.9%

Preproglucagon gene expression in ewes

This experiment was done to confirm *GCG* expression in the alimentary tract of ewes. Gastrointestinal tissue samples for rtPCR were scavenged from ewes in an experiment at Monash University (Ethics Approval: MARP-2014-054). Ewes were in good body condition with *ad libitum* access to food and water prior to, and at time of death and subsequent sample collection. Ewes had undergone oestrus synchronisation using a prostaglandin-based protocol and their stage of oestrous cycle recorded as either luteal (n =2) or follicular (n = 3, 16 hours post-prostaglandin injection).

Full thickness samples of gastrointestinal tissues were collected from the reticulum, rumen, omasum, abomasum, proximal and distal jejunum, proximal and distal ileum, proximal and distal colon and rectum. Samples were washed repeatedly in ice-cold, sterile saline before being snap frozen in dry ice and stored at -80°C until RNA extraction. The QIAzol Lysis Reagent standard protocol (Qiagen cat#79306) was used to extract RNA from the tissues. Following RNA extraction, cDNA was prepared and genomic DNA removed using QuantiTect Reverse Transcription Kit (Qiagen cat#205310). Real-time PCR reactions for each sample were set up in triplicate, with 50ng of cDNA using a QuantiNova SYBR Green Kit (Qiagen cat#208052) on the Rotor-Gene Q real-time PCR machine (95°C 2min; combined extension and annealing 60°C 1 min for 40 cycles) for all primer pairs. Purified DNA of known concentration was used as the assay standard in each instance. Optimisation of each primer

set was achieved by separation of PCR products by agarose gel electrophoresis followed by purification and sequencing to confirm identity (Table 1). The levels of expression of each mRNA and the estimated concentrations were determined relative to the standard preparation using Qiagen Rotor-Gene Q computer software. Similar amounts of RNA were used for each amplification and the ratio of each mRNA to the geometric mean of three reference genes (Cyclophilin, GAPDH and Malate Dehydrogenase (MDH1)) was calculated to correct for minor differences in the total amount of RNA used between samples.

Statistical analysis

Statistical analysis was performed using R (R Core Team 2018). For the purpose of analysis, progesterone values < 3nmol/L were considered consistent with the follicular phase, values > 3nmol/L consistent with the luteal phase.

Analysis used a multiple linear regression model for analysis of the effect of Exendin-4 on LH secretion. Logarithmic transformation of the data was performed after a diagnostic check of the model, to achieve homogeneity of variance. Model goodness-of-fit was determined using R-squared values and Akaike Information Criterion (AIC), which suggested a mixed effect model was most suitable.

LH pulse analysis was performed using a previously described method (Clarke, 1993) with a pulse defined as having a value higher than three times the assay S.D. of the preceding LH value. Values which fit the definition of a pulse but occurred concurrent with an LH surge were excluded from

analysis. Analysis was restricted to the period during which sampling occurred every 10 minutes. Inter-pulse interval (IPI) was calculated as the interval in time between the start of two adjacent pulses. A mixed effect regression model was fitted to the data for inferential analysis. Diagnostic check of the assumption conditions for the model suggested a logarithmic transformation was warranted.

An LH surge was defined as a monophasic rise in plasma LH concentration to a value ≥10ng/mL. The onset of a surge was the first value in a monophasic surge higher than three times the SD of the preceding LH value. Whether treatment had an effect on time of onset of surge was assessed using ANOVA, Kruskal-Wallis and Tukey HSD.

Plasma insulin and glucose concentration analysis was performed using two-way ANOVA and a linear regression model.

Analysis of the rtPCR results aimed to quantify the association between *GCG* expression using a primer for GCG, site of expression and phase of the oestrous cycle. An ordinary linear regression model was fitted using logarithmically transformed response variables and site and phase of the predictor values. The full factorial model was identified as the optimal model based on AIC.

In all cases, the “emmeans” function of the “emmeans” package in R was used for pairwise comparison (Lenth, 2022).

Results

Preliminary Study: effect of Exendin-4 on plasma LH concentrations during the luteal or follicular phases of the oestrous cycle

Infusion of Exendin-4 increased plasma LH (Fig 1) during and after infusion in both the Luteal and Follicular Phase groups ($p<0.001$). Mean plasma LH concentrations were higher in the Follicular Phase group during and after Exendin-4 infusion when compared to the Luteal Phase group ($p<0.01$ and $p<0.001$ respectively).

Main experiment: The effect of Exendin-4 on plasma LH concentrations in the Follicular Phase of the oestrous cycle.

Two ewes had high progesterone levels during the experimental period (12.6 and 16.9nmol/L respectively) and were excluded from all further analysis. A further three ewes experienced an LH surge during the first 3 hours of sampling and were also excluded from analysis of pulsatile LH secretion. Exendin-4 infusion increased mean LH concentrations in groups given 0.5mg and 2mg of the agonist ($p<0.001$) (Fig 2). This effect extended into the post-infusion period in both groups ($p< 0.001$ and 0.01 respectively) (Fig 2) although the effect diminished 1300 min post-infusion.

Exendin-4 infusion had no effect on inter-pulse interval (IPI), but increased pulse amplitude in both the 0.5mg and 2mg treatment groups compared to the saline control ($p< 0.001$) (Fig 3) The effect on amplitude extended into the post-infusion period ($p<0.001$). The increase in amplitude was greatest in the 2mg group ($p<0.001$), with no significant difference in amplitude between the control and 0.5mg group and the 0.5mg and 2mg groups.

The effect of Exendin-4 on the onset of the LH surge

Analysis of onset of LH surge demonstrated treatment had low predictive power (R-squared = 0.03).

Inferential analysis using ANOVA followed by TukeyHSD and Kruskal Wallis tests confirmed no discernible effect of Exendin-4 on onset of the LH surge. Observation of the data suggests Exendin-4 infusion coincident with the LH surge may have had a modulatory effect, as a protracted surge was seen in ewes in which this occurred (n=2) (Fig 4).

The effect Exendin-4 on plasma insulin and glucose concentrations

There was no effect Exendin-4 infusion on plasma insulin or glucose concentrations (Fig 5), but levels of insulin were higher in the 2mg group than in the control group throughout the experimental period ($p<0.05$).

Expression of the preproglucagon gene in the gastrointestinal tract

The level of expression of *GCG* was low in the forestomachs (reticulum, rumen, omasum and abomasum) and increased aborally, with greater ($p = <0.001$) expression in the jejunum, ileum, colon and rectum (Fig 6). Stage of oestrous cycle had no detectable effect on relative *GCG* expression.

Where they occurred, outliers in the data have been included in statistical modelling.

Discussion

This study confirms that an agonist of the GLP-1 receptor may stimulate the secretion of LH. We observed that systemic administration of Exendin-4 increased secretion of LH in ewes in both the follicular and luteal phase of the oestrous cycle (Fig 1). This finding is consistent with the general observation that anorexigenic pathways have a stimulatory effect on reproduction (Clarke, 2014). An

increase in LH secretion occurred with Exendin-4 doses of both 0.5mg and 2mg, with increased LH secretion typically starting during the infusion period (Figs 1 and 2). Interestingly, in our study, the effect on LH levels was not sustained into the period following infusion of either 0.5mg or 2mg of Exendin-4. This may reflect saturation of GLP-1 receptors or negative feedback of the supra-physiological dose of 2mg on the hypothalamus and/or pituitary but measurements of both glucose and insulin (*vide infra*) suggest that this was not the case.

Infusion of Exendin-4 increased LH pulse amplitude, without effect on inter-pulse interval (Fig3). This suggests the ewe's inherent GnRH pulse generator was already at its maximal intrinsic rate, as would be expected during the pre-ovulatory, follicular phase. Instead, Exendin-4 may have strengthened the signal from the hypothalamus to the pituitary to increase pulse amplitude without affecting the pulse interval. This is supported by our earlier work, which indicated that direct injection into the median eminence enhanced LH pulse amplitude. This suggests an effect on the release of GnRH at the level of the GnRH neuronal dendrites in the external zone of the ME (Arbabi et al. 2021)

We found no effect of Exendin-4 on timing of the preovulatory LH surge. This finding should be considered with caution, given the small sample size and individual variation that was apparent. Alternately, it is possible that GLP-1 may act without affecting the mechanisms generating the GnRH/LH surge e.g. on GnRH neuronal cell bodies or on pituitary gonadotropes, (Clarke 2018; Herbison 2018). Nevertheless, Exendin-4 treatment at or near the time of the LH surge may have had a modulatory effect, with a more protracted surge occurring in some treated ewes (n=2) (Fig 4).

A larger study is warranted to explore this effect. It would be expected, however, that GLP-1 infusion would not amplify the surge, since the pituitary stores of LH are depleted by 90% at the time of the surge in cyclic ewes (Roche et al. 1970).

The preliminary study found that hormonal state may influence the effect of GLP-1 or its agonists on LH secretion. Thus, although Exendin-4 infusion appeared to result in increased LH secretion in ewes during both the luteal and follicular phases of the cycle, the response was more pronounced and appeared to be more rapid in the ewes in the latter. This suggests sensitivity of the reproductive axis to GLP-1 is greatest during the early oestrous period, when serum progesterone levels are low and oestrogen levels high. This result is, perhaps, unsurprising, given progesterone exerts negative feedback effect on GnRH secretion through suppression of kisspeptin drive (Clarke 2014). The ability of Exendin-4 to overcome this restriction to some extent in our study is further evidence of the role of GLP-1 in reproduction. Indeed, all the ewes in the Luteal group had LH secretion below the assay sensitivity (<0.1ng/mL) until after the Exendin-4 infusion was commenced. The response to GLP-1 at different stages of the oestrous cycle could be due to either i) increased sensitivity of the reproductive axis to GLP-1 during the follicular phase or ii) increased expression of GLP-1 receptors in the hypothalamus and/or pituitary during the follicular phase. This could be due to either the increased concentration of oestrogen, low progesterone concentration or a combination thereof that occurs during the follicular phase. Inhibin, IGF-1 or other compounds (e.g. follistatin) could also be involved in regulation of the response to GLP-1. Outeirino-Iglesias et al. (2015) found that GLP-1 receptor

expression in the hypothalamus and pituitary varies during the oestrous cycle in the rat, but expression was greatest during diestrus and lowest during proestrus and oestrus, results which do not explain our observations in sheep and may indicate species differences. The results seen with our use of intact, cycling ewes, predominantly under the influence of oestrogen, compared with those in animals under the influence of progesterone may explain some differences in findings between studies, including the absence of an effect on pulse frequency seen in this study. Our findings suggest ovariectomised animals, male animals and females under the influence of progesterone may have a reduced or altered response to GLP-1 when compared with those under the influence of oestrogen.

We believe this is the first study to demonstrate that systemic administration of a GLP-1 receptor agonist can affect the reproductive axis at the level of the hypothalamus and/or pituitary. Further studies are required to define the level at which this effect is manifest. Both Exendin-4 and GLP-1 are able to penetrate the blood-brain barrier (Kastin & Akerstrom 2003) enabling action on kisspeptin and/or GnRH producing neurons to alter downstream LH secretion. Gut-derived GLP-1 or systemically administered Exendin-4 could also act outside the blood-brain-barrier at the level of the ME (Arbabi et al. 2021) or pituitary gland (Khan et al. 2022). The rapid LH response seen to Exendin-4 infusion in the present study tends to support the notion that GLP-1 influences GnRH secretion without entering the brain. One ewe appeared to respond during the period the infusion lines were being primed; perhaps due to inadvertent administration of the drug. The ME, which contains GnRH neurons, has a dense population of GLP-1 receptors (Larsen et al. 1997) and the increase in LH seen after micro-

injection of GLP-1/Exendin-4 into the ME suggests circulating GLP-1 most likely acts on the neurosecretory terminals of GnRH neurons this area (Arbabi et al. 2021). On the other hand, GLP-1 binding to rat pituitary is reported (Shimuzu et al. 1987), as is GLP-1R RNA expression (Khan et al. 2022; Outeirino-Iglesias et al. 2015) and action at this level cannot be excluded. As to whether gonadotrophs express GLP-1 receptor is unknown. Other studies of tissue distribution focussed on brain and tissues relating to metabolic function (Bullock et al. 1996; Campos et al. 1994).

The impact of a GLP-1 agonist or GLP-1 is not established with any degree of certainty. The paper of Outeirino-Iglesias et al. (2015) presents perplexing data which show that Exendin and GLP-1 have opposite effects in rats. These peculiar results require further interrogation. Other papers have reported effects on LH and testosterone secretion in human males.

Jeibmann et al (2005) infused GLP1 (0·4 pmol/kg/min, iv) into healthy men, observing no effect on gonadotrophin levels. Jensterle et al (2019) studied men with obesity related functional hypogonadism, comparing groups treated with either testosterone or Liraglutide, finding that the latter showed improvement of gonadotrophin levels. This study did not include a group of men receiving placebo alone. Izzi-Engbeaya et al (2020) infused (iv) 0.8 pmol/kg/min GLP-1 or placebo to healthy men and did not observe any effect on reproductive hormones, including gonadotrophins. There are no equivalent studies in women or other species, although GLP-1 receptor agonists are beneficial to treatment of polycystic ovarian syndrome (PCOS) in terms of weight loss and resumption of menses (Lamos et al 2017; Szeliga et al. 2022). Most notable is a study of IVF treatment of infertile,

obese PCOS women. In this study, supplementation with liraglutide to metformin treatment raised cumulative pregnancy rates over 12 months from 36% to 69%; gonadotrophin levels were not measured

Direct micro-injection of either GLP-1 or Exendin-4 increased LH pulse amplitude in the OVX ewe (Arbabi et al. 2021), strongly suggesting an effect to stimulate GnRH secretion at this level. On the other hand, Farkas et al. (2016) found that Exendin-4 had direct excitatory effect on GnRH neurons in brain slices from male mice, but the extent to which centrally produced or circulating moieties may exert an effect is an open question (Arbabi et al. 2021; Farkas et al. 2016; Heppner et al. 2017; Vastagh et al. 2021). The GLP1 receptor is expressed in the pituitary gland, ovary and uterus, a finding that suggests GLP-1 may act at multiple levels in the hypothalamic-pituitary-gonadal axis to regulate reproductive function (Khan et al. 2022; Outeirino-Iglesias et al. 2015). Nevertheless, plasma concentrations of GLP-1 in sheep (Relling et al. 2011) and cattle (Zapata et al. 2015) are in the picomolar range, so it is realistic to suppose that such levels may be physiologically relevant in terms of control of LH secretion. On the other hand, despite the adoption of the use of GLP-1 agonists for glycaemic control, there are no available data on the impact of such agents on gonadotrophin levels in humans. A recent review of the field (Zhao et al. 2021) considered the role that GLP-1 might play in polycystic disease but did not consider effects on gonadotrophins.

A study examining re-feeding of ewes after a period of feed restriction found restoration of LH pulsatility was associated with increased plasma insulin concentrations. It is possible, therefore, that

the incretin effect of GLP-1 is responsible for changes in LH response. Importantly, the lack of response of insulin and glucose to Exendin-4 infusion seen in our study demonstrates that the effect of GLP-1 on the reproductive axis is independent of its incretin effect, in ruminants at least.

To confirm that GLP-1 is produced in the gastrointestinal tract of the sheep, we used quantitative PCR and demonstrated that the pattern of *GCG* expression reflects that seen in other species, increasing aborally (Fig 6). Distribution of *GCG* expression in sheep is similar to that in mice and humans, where expression is greatest in the colon and rectum, whilst pigs and rats differ slightly, with expression greatest in the caecum and distal ileum respectively (Kuhre et al. 2014; Paternoster & Falasca, 2018). Presumably, GLP-1 is expressed via post-translational processing of *GCG* by intestinal L-cells in sheep, as is reported in other species (Holst 2007; Paternoster & Falasca 2018; Sandoval & D'Alessio 2015). As in other species, the conundrum exists between distribution of GLP-1 producing intestinal L-cells and the pattern of GLP-1 release, which occurs in a biphasic pattern following eating, consistent with ingesta being present in the stomach and small intestine (Dailey & Moran 2013). It is possible that GLP-1 produced centrally and by the small intestine is responsible for the relatively short-term effects of appetite suppression, incretin effect and the ileal brake. Thus, GLP-1 produced by the large intestine may constitute a baseline secretion responsible for longer-term effects of GLP-1 on reproduction, whilst also moderating glucose metabolism. Accordingly, the products of digestion in the large intestine typically reflects nutrition intake over a few days, rather than a single meal, which could influence the HPG axis. Interestingly, there was some indication that GLP-1 expression may

alter with stage of oestrous cycle, suggesting a reciprocal relationship between the reproductive axis and the gastrointestinal tract, but this requires confirmation with a larger sample size. Expression in the hindgut tended to be higher in the follicular phase, whilst the luteal phase was associated with greater foregut expression, but the small numbers limit statistical interpretation in this study. This may explain some of the differences in findings between our study and Johnson et al (2017), where *GCG* expression was greatest during pro-oestrus in the colon of the rat. That study also found an increase in plasma GLP-1 concentrations during pro-oestrus, suggesting that is when GLP-1 activity is greatest.

In conclusion, we have demonstrated that a GLP-1 agonist can stimulate LH secretion in female sheep when administered systemically. This response varies during the oestrous cycle, being more profound in the follicular phase of the cycle. This suggests that gut-to-brain signalling is a realistic phenomenon, in terms of reproductive function. Our preliminary observation that GLP-1 expression is greatest in the distal regions of the gastrointestinal tract prompt further studies on the expression of *GCG* expression and GLP-1 production in the colon and rectum and possible variation across the oestrous cycle.

Declaration of interest

The authors declare no conflict of interest which could prejudice the findings of this study.

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Author contribution

ES, IC, CJS and AG were involved in the design of the study. ES, IC, CJS, CPS and AG contributed to sample collection and experimentation. AR performed hormone assays and PCR laboratory work. ES performed data analysis and ES, AG, CJS, CPS and IC interpreted the results. ES drafted the paper and all authors read and finalized the manuscript.

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Figure Legend

Table 1: Primer sequences for gene of interest and reference genes for measurement of preproglucagon gene (*GCG)* expression from ovine intestinal samples. Nucleotides in bold are encoded on a separate exon from the remainder of the primer.

Figure 1. Plasma LH concentrations in the ewes of the preliminary study, treated with 2mg of Exendin-4 in either the follicular (n=4) or luteal phase (n=4) of the oestrous cycle. The data are presented as box plots, where the lower and upper margins of each box represent the 25th and 75th quartiles respectively. The solid lines within each box represent the median values. The vertical lines represent the minimum and maximum values of the data and dots represent outliers. The two solid lines mid-graph represent median values in the luteal phase group for which there were too few values to construct a box. ** $p<0.01$ compared to pre-infusion mean

Figure 2. Plasma LH concentrations in ewes in response to either vehicle (control), 0.5mg or 2mg of Exendin-4. The ewes were in the follicular phase of the oestrous cycle (n=11-12/group). The data are presented as box plots, as described for Figure 1. *** $p<0.001$ ** $p<0.01$ compared to pre-infusion means.

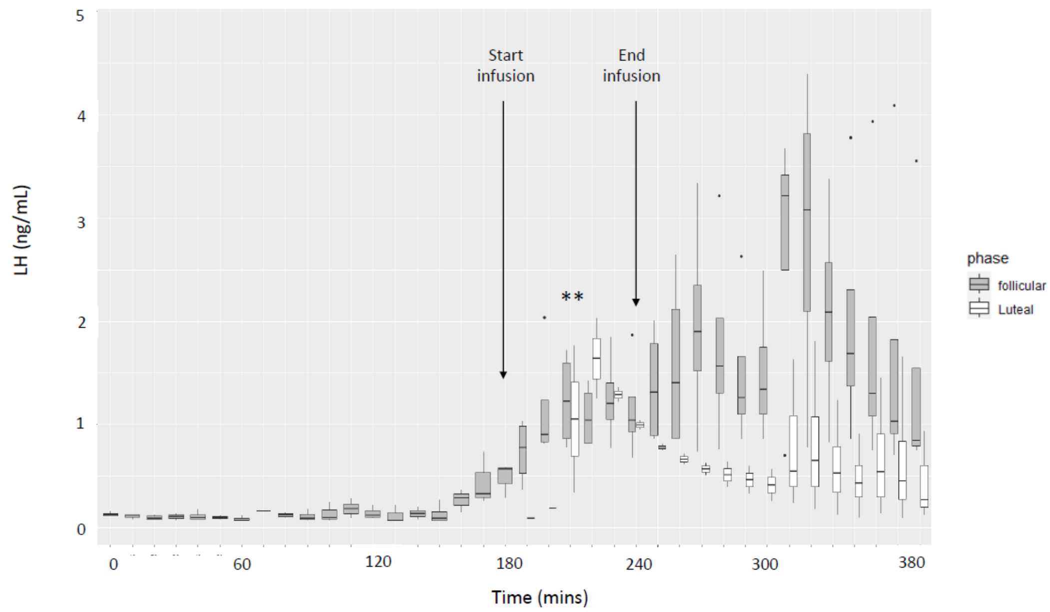
Figure 3. Mean ± SEM values for LH pulse amplitude and LH inter-pulse interval pre-infusion (t=-180-0mins), during infusion (t=0-60mins) and post-infusion (t=60-240mins) of infusion of Exendin-4 in the (a) vehicle (control), (b) 0.5mg Exendin-4 and (c) 2mg Exendin-4 (n=11-12/group). *** p <0.001 vs pre-infusion means

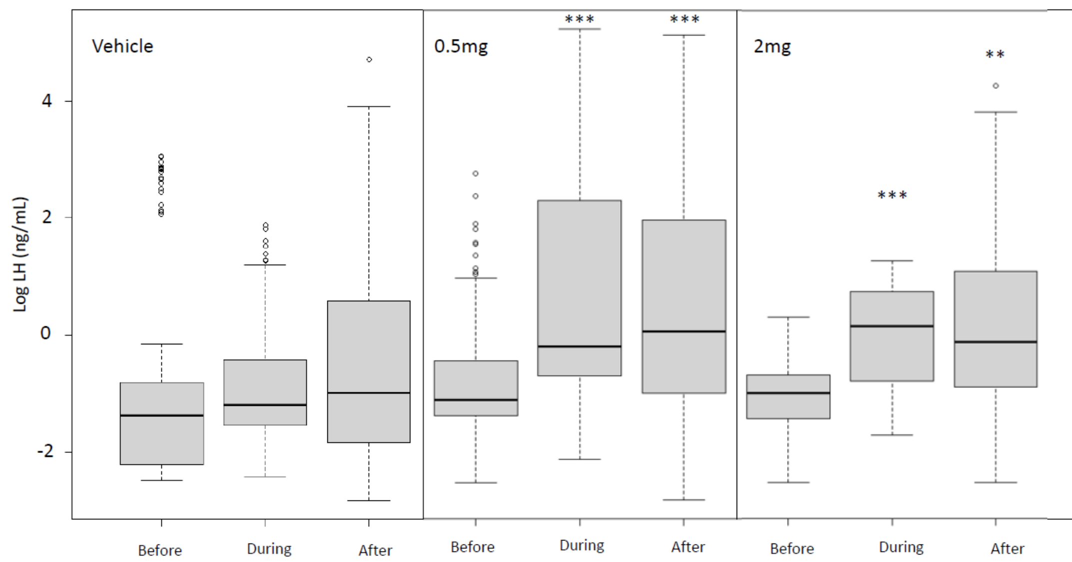
Figure 4. Examples of plasma LH surges in ewes receiving either vehicle (a), 0.5mg Exendin-4 (b) or 2mg Exendin-4 (c), when surges occurred during the time of infusion (beginning at t=0) in the main experiment, with ewe given vehicle for comparison.

Figure 5. Mean ± SEM concentrations of insulin and glucose in plasma pre-infusion (t=-180-0mins), during infusion (t=0-60mins) and post-infusion (t=60-240mins) of either vehicle (a) or 0.5mg Exendin-4 (b) or 2mg Exendin-4 (c) (n=11-12/group)

Figure 6. Relative *GCG* expression throughout the gastrointestinal tract of the ewe (n=5. Values for reticulum and proximal jejunum were n=4 and n=3 respectively). The data are presented as box plots, as described for Figure 1. *** p<0.001 vs expression in the reticulum.

Name	Accession Number	Forward Primer	Reverse Primer
GCG	AF529185	TCCAGTTCATTCCCAGCTCC	GTGAATGTGCCCTGTGAGTG
GAPDH	MN_001190390	TCAAGAAGGTGGTGAAGCAG	CCCAGCATCGAAGGTAGAA
Cyclophilin	JX534530	GCATACAGGTCCTGGCATCT	CATGCCCTCTTTCACTTTGC
MDH1	XM_004005845	ATCATTCTCGTGCTGTTGGA	CTTCTTTATCCGTGGCGATG

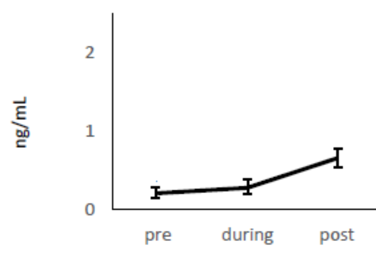


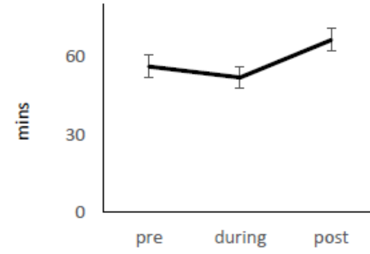


LH Pulse Amplitude

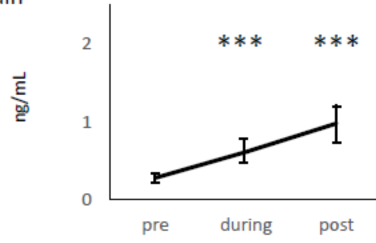
LH Inter-Pulse Interval

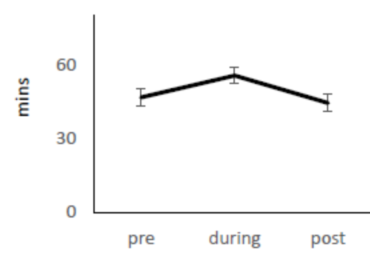
a) Vehicle



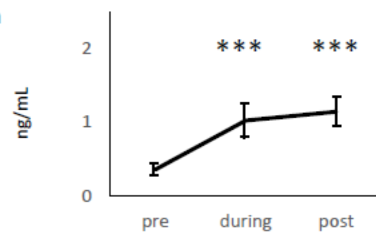


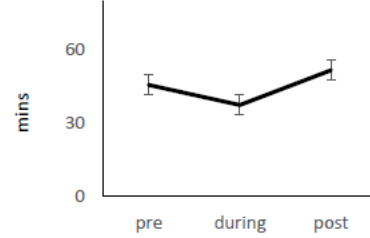
b) 0.5mg Exendin

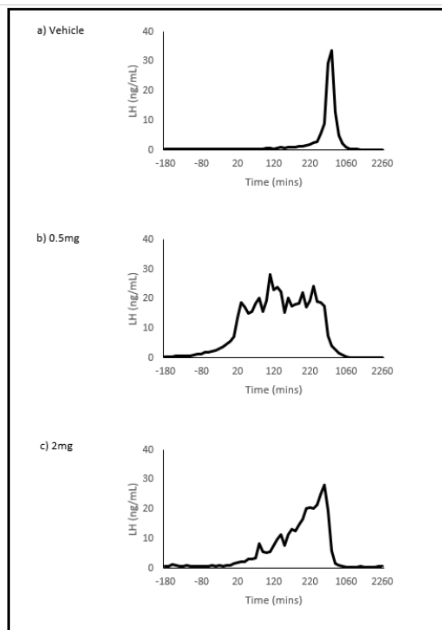


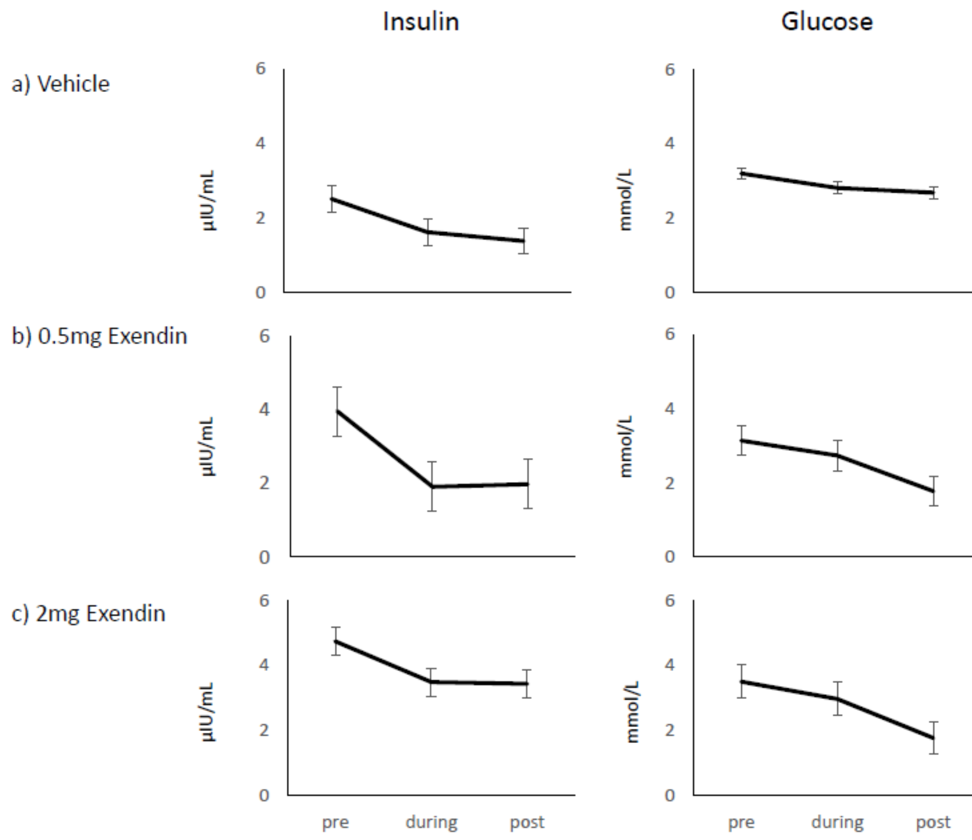


c) 2mg Exendin








Insulin
Glucose
a) Vehicle
b) 0.5mg Exendin
c) 2mg Exendin
µIU/mL
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0
2
4
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pre
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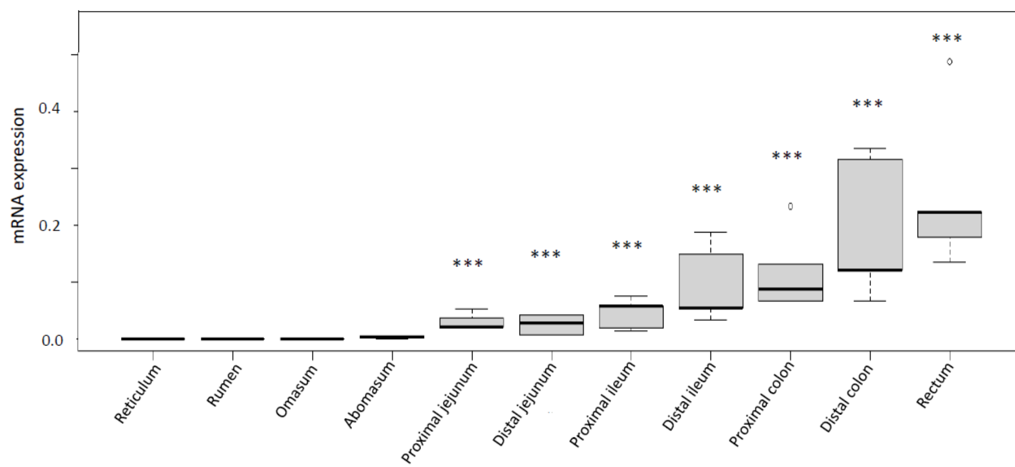
post


mRNA expression
0.0
0.2
0.4
***
Reticulum
Rumen
Omasum
Abomasum
Proximal jejunum
Distal jejunum
Proximal ileum
Distal ileum
Proximal colon
Distal colon
Rectum

Chapter 5: Infusion of the GLP-1 agonist, Exendin-4, does not alter FSH secretion in the follicular phase ewe

Research Chapter 5: Infusion of the GLP-1 agonist, Exendin-4, does not alter FSH secretion in the follicular phase ewe.

Preface

A preliminary study on the effect of a GLP-1 agonist, Exendin-4, on the reproductive axis in the ewe found that intravenous Exendin-4 infusion was associated with increased secretion of the gonadotrophs luteinizing hormone (LH) and follicle stimulating hormone (FSH). The effect of Exendin-4 on LH was confirmed in a larger study (Research chapter 4) which included a control group. This study was performed in tandem with that study, evaluating plasma FSH concentration rather than LH.

We found that plasma concentrations of FSH increased in all three experimental groups, including the vehicle control. This suggests that the increase in FSH secretion seen in the preliminary study was likely a result of an endogenous increase, rather than treatment induced.

Introduction

Glucagon-like peptide 1 is an anorexigenic peptide produced by intestinal L-cells and in the central nervous system in response to eating and the presence of ingesta in the small intestine (Huda et al., 2006). There is mounting evidence that GLP-1 also influences the reproductive axis. A peripherally administered GLP-1 agonist increased secretion of luteinizing hormone (LH) in ewes (Simpson et al 2023) whilst microinjection of GLP-1 and Exendin-4 into the ME increases LH secretion (Arbabi et al., 2021). Studies in rodents have found that GLP-1 receptor knock-out mice have delayed onset of puberty and that GLP-1 production varies during the oestrous cycle in rats (Johnson et al., 2017;

MacLusky et al., 2000). Administration of GLP-1 during pro-oestrus increased the amplitude of the pre-ovulatory LH surge in rats. Confusingly, the authors report that Exendin-4 had a different effect, decreasing the amplitude of the pre-ovulatory LH surge, although this contradictory effect has not been found in other studies, including sheep (Arbabi, Li, Henry, & Clarke, 2021, Outeirino-Iglesias et al., 2015).

The site of action of GLP-1 on the hypothalamic-pituitary-gonadal (HPG) axis is unknown, however there is growing evidence that GnRH neurons in the hypothalamus are an important target. Beak et al. (1998) found that GLP-1 stimulated GnRH release from hypothalamic cells *in vitro*. A high density of GLP-1 receptors are found in the arcuate nucleus (ARC) and median eminence (ME), areas of the hypothalamus which house GnRH neurons and terminals respectively and there are projections of GLP-1 producing neurons to the POA, where the majority of GnRH neurons are found in sheep (Göke, Larsen, Mikkelsen, & Sheikh, 1995; Larsen et al., 1997). Immunohistochemistry studies have shown GLP-1 immunoreactive fibres in close apposition to GnRH neurons (Farkas et al., 2016; Csaba Vastagh, Farkas, Scott, & Liposits, 2020) and GnRH neurons express the gene for GLP-1 receptors (Farkas et al., 2016). Exendin-4, a GLP-1 agonist has been found to stimulate GnRH neurons *in vitro* (Farkas et al., 2016).

Stimulation of GnRH neurons by GLP-1 would be expected to result in an increase in synthesis and/or secretion of both gonadotrophins, LH and FSH. GLP-1 could also have a direct effect on gonadotrophs, the gonads or (perhaps most likely) an effect at multiple levels of the hypothalamic-pituitary-gonadal

axis. To date, the relationship between GLP-1 and LH secretion has been better explored than that between GLP-1 and FSH (Arbabi et al., 2021; Beak et al., 1998; Jeibmann et al., 2005; Outeirino-Iglesias et al., 2015). Interestingly, Outeirino-Iglesias et al. (2015) found that GLP-1 treatment during pro-oestrus was associated with an increase in the number of antral follicles that developed and matured to ovulation, suggesting an effect of GLP-1 on FSH secretion. Exploring this relationship is important not only to understand the underlying physiology, but also to understand the effects of GLP-1 agonist effects on the reproductive axis, as they are being advocated as treatment for weight control and type 2 diabetes mellitus in humans (Madsbad, 2016). A long-acting form of the GLP-1 agonist semaglutide (Ozempic ®, Novo Nordisk Pharmaceuticals Pty. Ltd, Baulkham Hills NSW), colloquially termed the "skinny pen", has gained substantial media attention for its effect on weight (Hooten, 2023), although very little is understood about possible effects on reproduction, positive or otherwise. Interactions between GLP-1 and the HPG axis could be particularly useful in the treatment of polycystic ovarian syndrome (PCOS) in humans.

PCOS is a common cause of infertility in women, associated with increased LH secretion, decreased FSH secretion, insulin resistance, increased adrenal and ovarian hormone secretion (Lamos, Malek, & Davis, 2017; Moffett & Naughton, 2020). It is associated with, and exacerbated by, obesity but does occur in women of normal weight. A pilot study in obese women with PCOS found that the addition of the GLP-1 agonist liraglutide to metformin treatment improved pregnancy rate following IVF beyond what could be explained by weight loss and improved insulin resistance alone (Salamun, Jensterle,

Janez, & Vrtacnik Bokal, 2018). The authors theorised this could be due to improvement in endometrial function, however did not consider an effect on gonadotrophins. Other observational studies of PCOS, fertility and GLP-1 agonists have had similar findings (Elkind-Hirsch et al., 2008; Jensterle et al., 2019). Exendin-4 was associated with a return to normal cyclicity that preceded changes in insulin resistance in overweight women with PCOS. Several women were excluded from that study after becoming pregnant during treatment (Elkind-Hirsch et al., 2008). The increasing evidence that GLP-1 dysfunction is a symptom or a causative factor in development of PCOS regardless of obesity further supports a link between GLP-1 and reproductive (dys)function and should make such findings unsurprising (Ferjan, 2019; Lamos et al., 2017; Moffett & Naughton, 2020; Vrbikova et al., 2008). However, most clinical studies do not consider nor measure gonadotrophin response to treatment with GLP-1 agonists and we must instead rely on animal models to better understand such relationships. Improved FSH secretion, particularly, would be expected to improve the effects of PCOS on cyclicity, oocyte quality and retrieval and may contribute to the improved conception and pregnancy rates observed after exposure to GLP-1 agonists.

The results from our pilot study (see Research Chapter 3) found that both FSH and LH secretion increased after an infusion of 2mg of the GLP-1 agonist Exendin-4 in the luteal and follicular phases of the oestrous cycle in mature ewes. We have since confirmed that Exendin-4 infusion results in increased LH secretion in a larger study using ewes in the follicular phase. This study aimed to observe

the effect of Exendin-4 infusion on FSH secretion in ewes in the follicular phase of the oestrous cycle and was conducted in tandem with our study on LH secretion (Research Chapter 4).

Materials and methods

This study was performed in two replicates in the natural breeding season of sheep (Summer/Autumn) in consecutive years.

Animal care and ethics statement

This study protocol was approved by the Charles Sturt University Animal Care and Ethics Committee, approval numbers A19005 and A19385.

Animals and experimental design

The experimental design, including animal selection, housing, oestrus synchronisation, jugular catheterisation, sampling procedure, Exendin-4 infusion and monitoring post-procedure is described in Research Chapter 2.

In brief, 5 or 8 year old ewes in body condition score 2.5/5 were randomly allocated to either the vehicle control, 0.5mg or 2mg experimental group (n= 4 ewes per group). All ewes underwent oestrus synchronization such that they were in the follicular phase of the oestrous cycle on the day of infusion and sampling, which was confirmed with plasma progesterone assays. Venous blood samples were collected from an indwelling jugular catheter every 10 minutes from t= -180mins to t=240mins, relative to the start of infusions. Then, 3 consecutive samples were taken at 30min intervals, followed

by sampling at 2h intervals until t=2260mins. Infusion of either 0.5mg Exendin-4, 2mg Exendin-4 or the vehicle control was performed over a 60-minute period, beginning at t=0.

Table 5.1 Live weight, treatment group, serum progesterone concentration and sampling notes for ewes recruited into the main study

EWE	WEIGHT (KG)	GROUP (MG/ML)	P4 (NMOL/L)	NOTES
A12	57	0	12.6	High P4. Excluded from analysis
A22	53.5	0.5	1.4	
A32	43.5	2	0.7	
B12	53	0.5	16.9	5-6 weeks gestation. Excluded from analysis
B22	44.5	2	1.7	
B32	46	0	1.2	
C12	47.5	0	0.9	
C22	54	2	1.1	
C32	56	0.5	1	
D12	53	0.5	0.7	
D22	57	0	1.1	
D32	54	2	1.2	
A13	55	2	2	
A23	51.5	0.5	1.5	
A33	55.5	0	1.4	
B13	53.5	2	1.5	
B23	50	0.5	2.1	
B33	52.5	0	1.8	
C13	55	0	1.4	
C23	58	2	2.4	
C33	57	0.5	1	
D13	54.5	0	1.5	
D23	56	0.5	0.9	
D33	57.5	2	1.9	

Serum progesterone sampling and assay

A single 3-5mL blood sample was collected from the sampling line into a plain (red top) vacutainer immediately prior to the sampling period, for progesterone assay. Sampling technique was as described below. Samples were analysed the same morning at the Charles Sturt University Veterinary Diagnostic laboratory using solid phase, competitive immunoassays (IMMULITE 1000, Siemens Healthcare, Hawthorn East, VIC, Australia).

FSH Radioimunnoassay

Blood samples were centrifuged (4000 rpm for 10 min) and plasma was aspirated from the top of the sample into a separate tube and stored at -20°C. Samples were maintained at-20°C until they were shipped frozen for analysis of FSH concentrations.

Ovine plasma FSH concentrations were measured in a homologous double antibody radioimmunoassay (RIA) (Bremner, Findlay, Lee, de Kretser, & Cumming, 1980)

performed using FSH antiserum raised in rabbit (NIDDK-anti-oFSH-1). Ovine FSH (NHPP-oFSH-SIAFP-RP-2) was used as standard and highly purified ovine FSH (NHPP-USDA-oFSH-19-SIAP) was used for iodination.

For the assays, 200µl or 100µl assay buffer (0.5% BSA/0.03M sodium phosphate monobasic/0.2M sodium phosphate dibasic/0.1% sodium azide/0.1% triton X), 200ul first antibody (1:40,000) diluted in 1:2000 normal rabbit serum (NRS) and 100µl ^{125}I iodinated ovine FSH (15,000cpm/100µl) was added

to duplicate plastic tubes containing either 100µl standard (2.5-320 ng/ml) or 200µl ovine plasma. After preincubation at 32°C for 24 h, the antibody bound hormone was separated from the free hormone by the addition of 200µl goat-anti-rabbit serum (1:600). The tubes were incubated with the second antibody overnight at 32°C before centrifugation (3500 rpm, 30min, 4°C), after which the supernatant was aspirated, and the precipitate counted in a gamma counter. The computation of assay data for determination of FSH concentrations was conducted using the statistical program of H. G. Burger, Lee, and Rennie (1972).

Intra-assay coefficient of variance (CV) was less than 10% between 0.20 and 34.36ng/mL in the first replicate and between 0.34 and 26.55ng/mL in the second replicate.

Statistical analysis

Statistical analysis was performed using R (R Core Team 2018). For the purpose of analysis, progesterone values < 3nmol/L were considered consistent with the follicular phase, values > 3nmol/L consistent with the luteal phase.

Every fifth sample collected was used for FSH analysis, beginning with the first sample taken (t=-190 or t=-180 depending on replicate), giving a total of 13 samples tested per ewe (n=22 ewes) across the entire experimental period

A multiple linear regression model was used for analysis of the effect of Exendin-4 on FSH secretion. Logarithmic transformation of the data was performed after a diagnostic check of the model, to

achieve homogeneity of variance. The “emmeans” function of the “emmeans” package in R was used for pairwise comparisons (Lenth, 2022).

Results

Two ewes had high progesterone levels during the experimental period (12.6 and 16.9nmol/L respectively) and were excluded from all further analysis (Table 5.1).

FSH assay sensitivity was 0.045ng/mL. Mean plasma FSH samples in the vehicle control group were 0.4ng/mL pre-infusion and 0.6ng/mL post-infusion. Mean plasma FSH samples in the 0.5mg group were 0.3ng/mL pre-infusion and 0.6ng/mL post-infusion. Mean plasma FSH samples in the 2mg group were 0.4ng/mL pre-infusion and 0.6ng/mL post-infusion. Plasma FSH concentration was increased post infusion of Exendin-4 in all three experimental groups ($p<0.05$). There was no statistical difference in plasma FSH concentration between groups pre- or post- infusion (Fig 5.1).

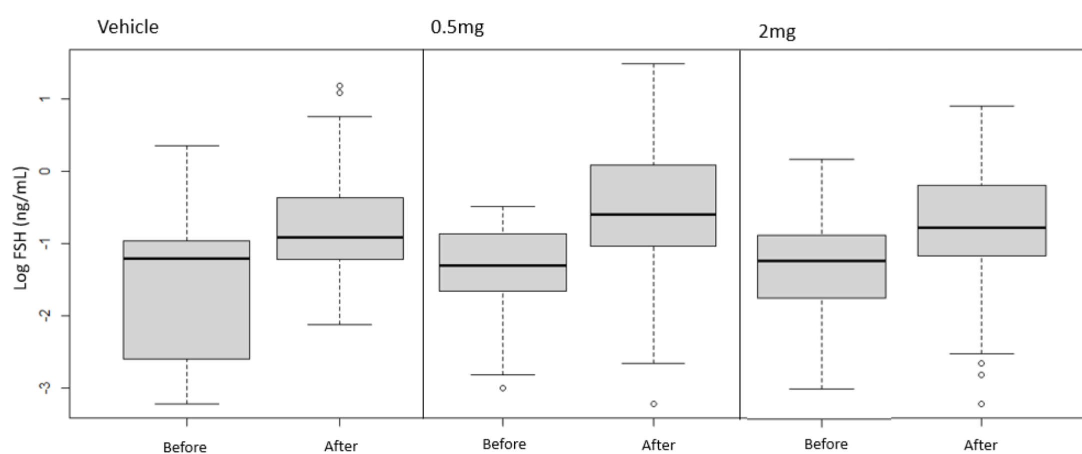


Figure 5.1 Boxplot of FSH response to treatment of ewes with vehicle (control), 0.5mg or 2mg of Exendin-4 at each phase of the main study, presented on a logarithmic scale.

Discussion

This study found plasma FSH concentrations were increased following exposure to the GLP-1 agonist, Exendin-4, however the increase in secretion was similar across all three treatment groups including the saline control. This suggests that any effect seen was not a result of a GLP-1 effect but instead an endogenous increase related to the stage of oestrous cycle or the oestrus synchronization protocol that was used. The oestrus synchronization protocol was progesterone and prostaglandin based, with no GnRH, LH or FSH analogues eg equine chorionic gonadotrophin (eCG) used. Generally, exposure to progesterone favours FSH secretion as it reduces the frequency of GnRH pulsatility, increasing baseline secretion of FSH (Pak & Chung, 2011), although this relationship is not as strong in the ewe. Instead, direct inhibition of oestradiol and inhibin on gonadotrophs appears to be the primary means of regulation of FSH (Baird & McNeilly, 1981). In other species, the rate of clearance of FSH is also thought to be decreased under the influence of progesterone (Pak & Chung, 2011). The cessation of progesterone exposure and administration of PGF2α and subsequent increased effect of oestrogen and inhibin would then be expected to result in either no change or a decrease in baseline FSH secretion, rather than the increase seen.

Instead, it seems more likely that we observed an endogenous increase in FSH secretion in this study related to the stage of oestrous cycle. The oestradiol-induced GnRH surge which occurs in the follicular phase results in the pre-ovulatory LH surge (Clarke, 2018) and is associated with increased FSH secretion, although the increase is small in comparison to the increase in LH secretion (Baird & McNeilly, 1981; Bartlewski et al., 1999). This rise in FSH secretion is thought to be due to a decrease in oestrogen and inhibin secretion from the pre-ovulatory follicle (Baird, 1978). A second increase in FSH occurs approximately 20-36 hours later, following ovulation and the removal of inhibitory factors secreted by the pre-ovulatory ovarian follicle (Baird & McNeilly, 1981). Our experimental design aimed for the ewes to be in the follicular phase and "capture" the pre-ovulatory LH surge (see Research Chapter 4). It seems very likely then that we captured the first of these two periods of increased FSH secretion. It is interesting that in the pilot study there was also increased secretion of FSH in ewe in the luteal phase of study. A transient increase in FSH secretion, associated with follicular wave emergence, occurs on day 5-6 of the oestrous cycle (Bartlewski et al., 1999) and it is possible that this increase was occurring during the pilot study. Importantly, there was no control group used in the pilot study and so we are unable to determine whether what was observed in that study was an effect of treatment or not.

Although there was no detectable effect of GLP-1 on FSH secretion in this study, this does not mean such a relationship does not exist. The synthesis of the FSH β sub-unit is GnRH-dependent (Burger et al., 2004). The strong temporal relationship which exists between GnRH input and LH, whereby a

GnRH pulse is followed almost immediately by an LH pulse downstream, (Clarke & Cummins, 1982) is not the same for FSH secretion. Secretion of FSH is directly related to the releasable pool of FSH in the pituitary (Clarke et al., 2002). The relatively long half-life, high basal secretion, presence of different isoforms and continued secretion following withdrawal of GnRH input mean plasma FSH concentrations do not directly reflect changes in GnRH secretion (Clarke et al., 2002). Oestradiol and peptides from the antral follicle (activin, inhibin, follistatin) also have a direct, regulatory effect on FSH secretion from the pituitary and have the potential to obscure GnRH-driven change (Orisaka et al., 2021; Padmanabhan & Cardoso, 2020). Further study is required to determine whether GLP-1 has a stimulatory effect on FSH secretion as it does on GnRH and LH secretion. The nature of FSH secretion and findings of this study suggests that the use of gonad-intact females is not an ideal study population for studies on FSH secretion. Instead, ovariectomized (OVX) ewes or ewes under a hormonal "clamp" may be better candidates for study. The relatively long lag time for changes in GnRH secretion to be reflected in FSH secretion and long half-life suggests a longer, less frequent sampling regimen may be more appropriate (Clarke et al., 2002). Alternately, molecular techniques such as in situ hybridization and qPCR to observe whether exposure to GLP-1 has an effect on GnRH receptor expression or FSH β subunit expression in the pituitary could be illuminating.

Although the observation by Salamun et al. (2018) that liraglutide improved IVF outcomes in women with PCOS is suggestive of an effect on FSH secretion and follicular dynamics, the findings of our study do not support this. It may be that liraglutide has beneficial effects on the endometrium, rather than

affecting endocrinology, as those authors suggested. Alternately, GLP-1 may have more general beneficial effects on reproduction which improve fertility outcomes, including but not limited to changes in gonadotrophin secretion. Treatment with GLP-1 agonists seems likely to be of particular benefit in conditions where derangements in glucose metabolism contribute to poor fertility, such as PCOS, diabetes mellitus, obesity and insulin resistance.

Conclusion

In contrast with our pilot study, this study found no effect of treatment with a GLP-1 agonist on FSH secretion, with previous observed effects likely due to an endogenous increase independent of Exendin-4 exposure. More tailored studies are required to determine if GLP-1 affects FSH secretion as it does LH and whether this is due to increased upstream secretion of GnRH from the hypothalamus.

Chapter 6: Preproglucagon gene expression in the gastrointestinal tract of the ewe.

Research Chapter 6: Preproglucagon gene expression in the gastrointestinal tract of the ewe

Preface

The preproglucagon gene is responsible for the production of several metabolically active peptides including glucagon, glicentin, oxyntomodulin and GLP-1 and GLP-2. Expression of the preproglucagon gene by intestinal L-cells results in the production of GLP-1. GLP-1 is known to be metabolically active in sheep, however expression of the preproglucagon gene and the presence of L-cells has not been confirmed. The distribution of preproglucagon gene expression is not well reported in domestic mammals, with the cow, cat, pig and rodent the only species in which distribution is reported. To date, the possibility of GLP-1 production in the intestine being altered by reproductive status has only been studied in rodents.

Samples of gastrointestinal tract from the reticulum to the rectum were collected from ewes (n=5) of known stage of oestrous and qPCR performed to determine preproglucagon gene expression. This study confirms the expression of the preproglucagon gene in ovine intestine using qPCR and found that expression increases aborally, similar to other species. Small sample size prevented findings relating to stage of oestrous cycle and preproglucagon gene expression.

Introduction

Glucagon-like peptide 1 (GLP-1) is a 30 amino acid peptide produced by post-translational processing of the preproglucagon gene (Huda et al., 2006; Sekar, 2017). It was the sequencing of the

preproglucagon gene in the 1980's which led to the discovery that the gene which encodes glucagon also produced two glucagon-like sequences; glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2) (Holst, 2007). The preproglucagon gene is expressed by three main populations of cell types; pancreatic α-cells, intestinal L-cells and centrally in neurons of the nucleus of the solitary tract (Larsen et al., 1997). The end product secreted is determined by which post-translational pathway is present. The Psck-2 post-translational pathway is dominant in pancreatic α-cells and produces glucagon, glicentin-related pancreatic peptide, intervening peptide 1 and the major proglucagon fragment (Holst, 2007). The Psck-1/3 pathway results in the formation of GLP-1, GLP-2, oxyntomodulin, glicentin and intervening peptide 2 and is expressed centrally in the brain and in intestinal L-cells (Holst, 2007).

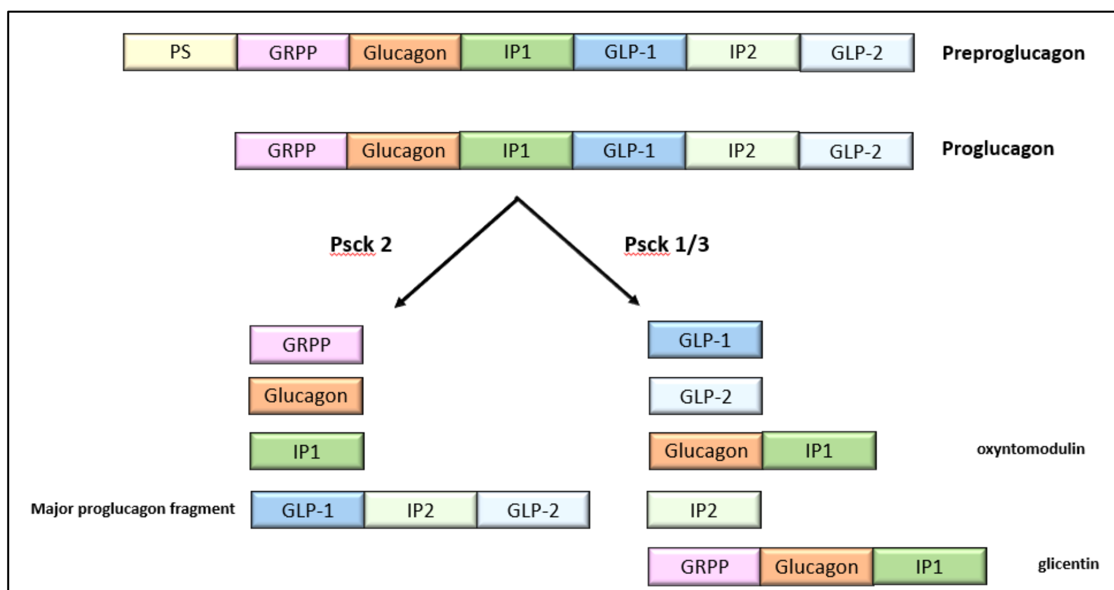


Figure 6.1: Posttranslational processing of the preproglucagon gene, showing the Psck2 and Psck 1/3 pathways. Adapted from Sandoval & D'Alessio, 2015.

Intestinal-L cells, a subset of enteroendocrine cells, are morphologically similar to pancreatic α-cells, with large intra-cellular granules (Holst, 2007). These cells are located throughout the intestinal tract, although in most species their density is reported to be greatest in the ileum and colon (Holst, 2007).

Generally, the preproglucagon gene and effects of GLP-1 are well preserved across mammalian species (Holst, 2007; Irwin, 2001). Most studies have focused on monogastric species and there are substantial gaps in our understanding of the physiology of GLP-1 in ruminants, with considerable extrapolation from other species (Thanthan et al., 2012). There are substantial differences between the ruminant digestive tract, with its well developed, four-chambered "stomach" capable of extensive fermentation, and that of monogastrics, which make such extrapolation problematic. Ingesta release from the ruminant stomach is slow and gradual, rather than episodic, and intake is often prolonged, reflecting patterns of grazing. Upon exit from the abomasum, the ingesta is vastly different from when it was consumed, having undergone anaerobic fermentation, and contains a relatively large amount of microbes, microbial by-products and proteins.

Observational studies suggest the pattern of release and effect of GLP-1 in ruminants is similar to monogastrics (El-Sabagh et al., 2015; El-Sabagh et al., 2016; Faulkner & Martin, 1997). GLP-1 is secreted in response to eating, reduces dry matter intake and has an incretin-like effect in ruminants (El-Sabagh et al., 2015; Fukumori, 2018). Glucose and lipids are the main regulators of GLP-1 release in monogastric species, but do not have the same effect in ruminants (Faulkner & Martin, 1997;

Relling et al., 2011). Instead, short chain fatty acids (SCFAs) and volatile fatty acids (VFAs) stimulate GLP-1 secretion (Faulkner & Martin, 1997; Fukumori, 2018; Hatew et al., 2019). This is not surprising when considering that ruminal fermentation produced VFAs, not glucose, are the primary energy source in ruminants (Hatew et al., 2019; Pond, 1995).

Despite these differences, it is generally assumed that intestinal L-cells are the primary source of GLP-1 production and secretion in ruminants, as it is in other species. To the author's knowledge, there are no studies to confirm this, although Taylor-Edwards et al. (2010) and Connor et al. (2010) have demonstrated expression of the preproglucagon gene in the bovine intestinal tract.

There is increasing evidence that GLP-1 has a stimulatory effect on reproduction (Heppner et al., 2017; Khan et al., 2022; Outeirino-Iglesias et al., 2015; Csaba Vastagh et al., 2020). There is also some evidence that this relationship may be reciprocal, with reproductive status altering GLP-1 production and function. Johnson, Saffey, and Taylor (2017) found that total GLP-1 concentration in plasma and colon tissue varied during the oestrous cycle of the rat, with concentrations in pro-oestrus and metoestrus increased when compared with dioestrus. Plasma concentrations of GLP-1 and other gut peptides increased after calving in dairy cows, when progesterone concentrations rapidly decrease (Relling, 2007). There appears to be a direct effect of oestrogen on intestinal L-cells, which increases GLP-1 production and secretion (Handgraaf & Philippe, 2019). Ovariectomised rats had a decreased GLP-1 response to oral glucose compared to intact controls (Handgraaf, Dusaulcy, Visentin, Philippe, & Gosmain, 2018) whilst women had a better GLP-1 response to glucose than men. This indicates that

hormones related to the reproductive cycle, likely progesterone (P4) and oestrogen (E2) have a direct effect on GLP-1 secretion.

The aim of this study was to confirm expression of the preproglucagon gene in ovine intestine by using quantitative PCR of GLP-1 mRNA to allow comparison between gastrointestinal sites. We also included a preliminary study to analyse whether stage of oestrous cycle influences expression.

Materials and methods

Samples for this study were collected from a separate project at Monash University, Australia (MARP-2014-054) in which euthanasia was the study end-point. Ewes had undergone oestrus synchronisation using a prostaglandin-based protocol and their stage of oestrous cycle recorded as either luteal (n =2) or follicular (n = 3, 16 hours post prostaglandin injection). Ewes were in good body condition with *ad libitum* access to food and water prior to, and at time of death and subsequent sample collection.

Full thickness samples of gastrointestinal tissue were collected from 11 sites from each ewe following euthanasia and washed repeatedly in ice-cold, sterile saline before being snap frozen in dry-ice and stored at -80°C until RNA extraction. Samples were collected from the reticulum, rumen, omasum, abomasum, proximal and distal jejunum, proximal and distal ileum, proximal and distal colon and rectum.

Quantitative PCR was performed at Monash University. The Qiazol Lysis Reagant standard protocol (Qiagen cat#79306) was used for RNA extraction from tissue. A QuantiTect Reverse Transcription Kit

(Qiagen cat#205310) was then used for preparation of cDNA and genomic DNA removal. Real-time PCR reactions for each sample were set up with 50ng of cDNA, in triplicate, using a QuantiNova SYBR Green Kit (Qiagen cat#208052) on the Rotor-Gene Q real-time PCR machine (95°C 2min; combined extension and annealing 60°C 1 min for 40 cycles) for all primer pairs (Table 6.1). Purified DNA of known concentration was used as the assay standard. Optimisation of each primer set involved the separation of PCR products by agarose gel electrophoresis, followed by purification and sequencing to confirm identity. Qiagen Rotor-Gene Q computer software was used to determine the levels of expression of each mRNA and estimated concentration relative to the standard. Similar amounts of RNA were used for each amplification and the ratio of each mRNA to the geometric mean of three reference genes (Cyclophilin, GAPDH and Malate Dehydrogenase (MDH1)) was calculated to correct for minor differences in the total amount of RNA used between samples.

Table 6.1 Primer sequences for gene of interest and reference genes for measurement of preproglucagon gene (*GCG)* expression from the gastrointestinal tract of the ewe

Name	Accession Number	Forward Primer	Reverse Primer
GCG	AF529185	TCCAGTTCATTCCCGGCTCC	GTGAATGTGCCCTGTGAGTG
GAPDH	MN_001190390	TCAAGAAGGTGGTGAAGCAG	CCCAGCATCGAAGGTAGAA
Cyclophilin	JX534530	GCATACAGGTCCTGGCATCT	CATGCCCTCTTTCACTTTGC
MDH1	XM_004005845	ATCATTCTCGTGCTGTTGGA	CTTCTTTATCCGTGGCGATG

Statistical analysis

Statistical analysis was performed using R (R Core Team 2018). Corrected mRNA expression values were used for analysis (see above). Data analysis aimed at quantifying the relationship between mRNA expression and two predictor variables; “site” and “phase”. Results are reported relative to the assay standard.

Inferential analysis was performed using log transformed mRNA expression values to fit an ordinary linear regression model. The full factorial model was identified as the optimal model based on Akaike Information Criterion (Crawley, 2013). Adjusted R-squared values were used to indicate model goodness of fit. The lmer() function in R package ‘lme4’ was used to fit the full factorial mixed effect linear regression model. However, because of the very small sample size (n=5), the mixed effect model did not computationally converge (the resulting model parameter estimation results were not reliable) so instead the lm() function was used to generate a linear model for multiple comparison analysis after confirming that estimated standard errors were the same as in a linear mixed model (lmm() function).

The emmeans() function of the emmeans package was used to estimate the 95% confidence intervals for the marginal means and allow pairwise comparison between site and phase at different levels. Anti-log transform was used when needed to report outcomes.

Results

Expression of the preproglucagon gene was found in all samples, although relative expression was <0.001 in the reticulum, rumen and omasum. Samples were missing from the proximal jejunum in two ewes and one from the reticulum, both from the follicular group. The study design was not balanced, due to the different number of ewes in each group and missing samples. The adjusted R-squared value was 0.9449.

Table 6.2 Mean, median and standard deviation of relative *GCG* expression from different sites of the gastrointestinal tract of the ewe

	Mean	Median	SD
reticulum	0	0	0
rumen	0	0	0
omasum	0	0	0
abomasum	0.004	0.004	0.003
proximal jejunum	0.033	0.022	0.018
distal jejunum	0.026	0.029	0.018
proximal ileum	0.045	0.058	0.027
distal ileum	0.097	0.056	0.068
proximal colon	0.118	0.089	0.07
distal colon	0.192	0.121	0.124
rectum	0.249	0.223	0.138

Relative expression was low (<0.004) in the forestomachs (reticulum, rumen, omasum and abomasum) and increased aborally, with expression greater ($p \leq 0.001$) in the jejunum, colon and rectum compared to the reticulum (Fig 6.2). Median relative expression was greatest in the rectum (0.223, followed by the distal colon, proximal colon, distal ileum, proximal ileum, distal jejunum,

proximal jejunum, abomasum, reticulum and rumen. Relative expression was lowest in the omasum (<0.001) (Table 6.2).

Median relative expression for the follicular and luteal phase across all sites was 0.025 and 0.043 respectively, whilst mean relative expression was 0.085 and 0.054. There was no significant difference in overall mRNA expression between the luteal and the follicular groups, although expression trended higher in the luteal group ($p < 0.1$) (Fig. 6.3).

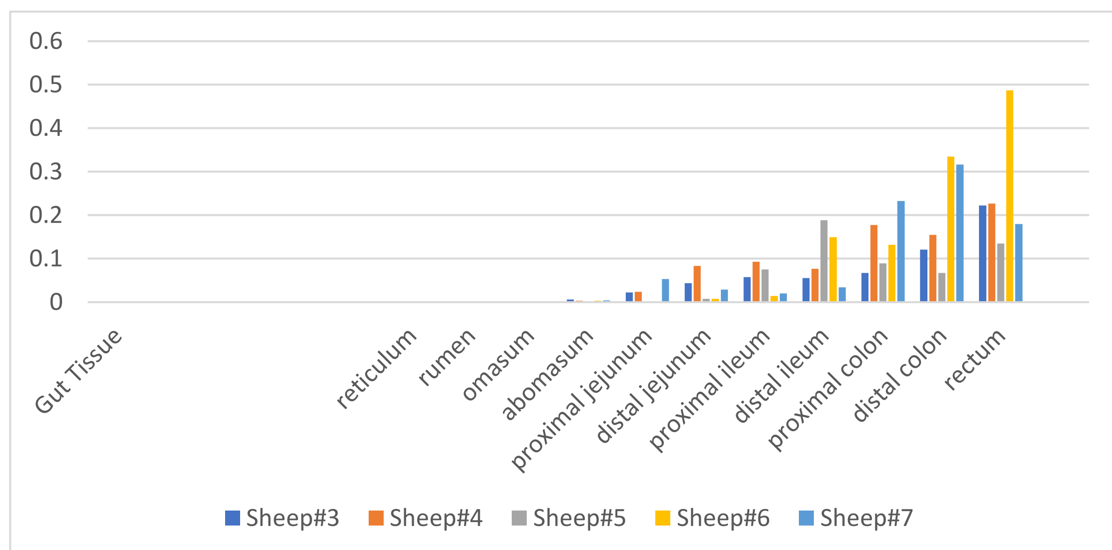


Figure 6.2 Histograph of relative *GCG* expression at different sites along the gastrointestinal tract of the ewe

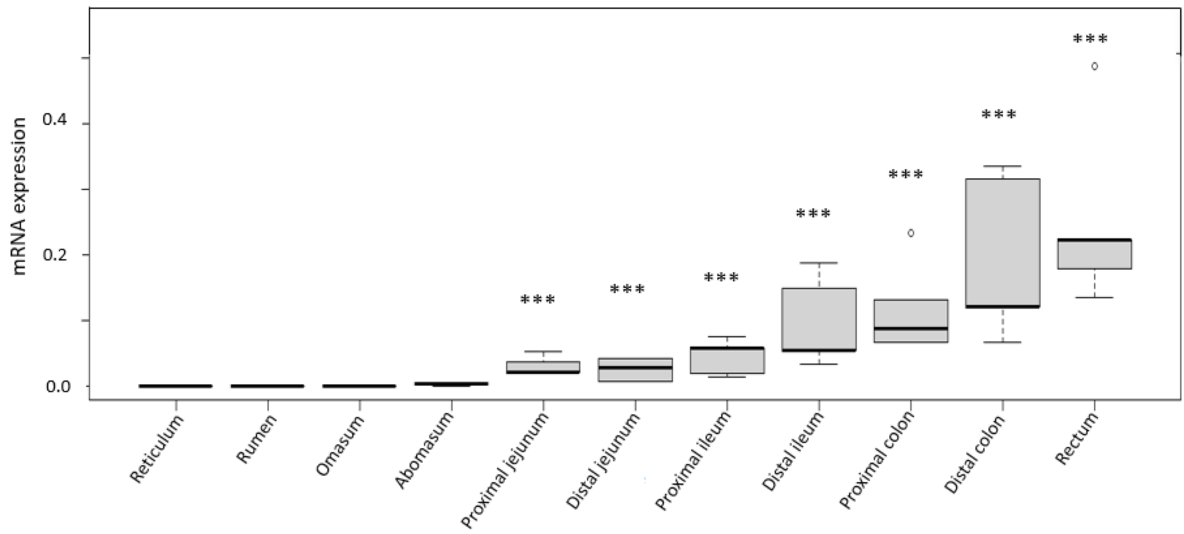


Figure 6.3 Boxplot of relative *GCG* expression at different sites along the gastrointestinal tract of the ewe.

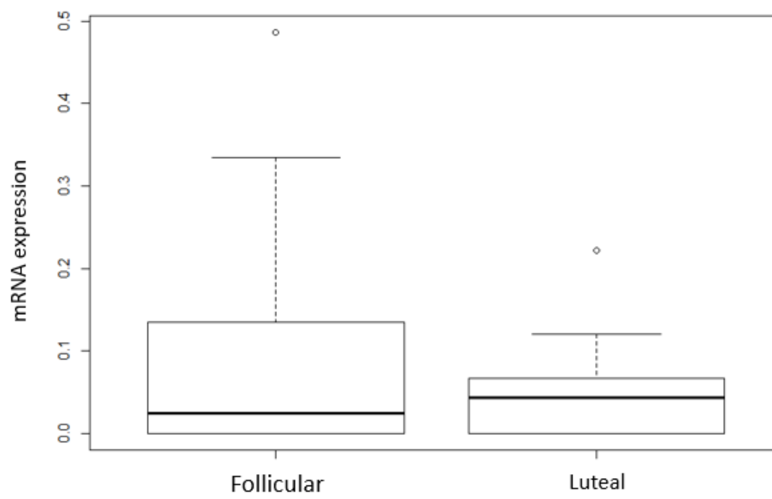


Figure 6.4 Boxplot of relative *GCG* expression of ewes in either the follicular phase and luteal phase of the oestrous cycle

Discussion

This study confirms, for the first time, that the preproglucagon gene is expressed in the ovine gastrointestinal tract. Similar to PCR studies of the preproglucagon gene in cattle (Connor et al., 2010; Pezeshki, Muench, & Chelikani, 2012), we found that expression of GLP-1 was very low in the forestomachs and increased aborally, with expression greatest in the rectum and colon. This finding is consistent with studies in monogastric species, where the distal gastrointestinal tract is generally cited as the having the greatest concentration of GLP-1, GLP-1 secreting intestinal L-cells, or preproglucagon gene expression. Distribution of GLP-1 is reportedly highest in the distal ileum in rats, distal colon and rectum in mice, ileum and colon in cats and rectum in humans (Kuhre et al., 2014; Paternoster & Falasca, 2018). Pigs are reported to have greatest concentration of GLP-1 production in the caecum, which is the site of fermentation in this species. This has led some authors to suggest that intestinal L-cell distribution and GLP-1 production may reflect the microbiota and products of microbial digestion (Covasa, Stephens, Toderean, & Cobuz, 2019; Paternoster & Falasca, 2018). However, our findings in sheep, are at odds with this theory, as expression of GLP-1 is low in the ruminant forestomachs and jejunum, where the microbial population and products of microbial fermentation are greatest. This observation highlights the importance of understanding L-cell function and how it may differ between species, rather than relying on extrapolation from rodents, whose digestive physiology is not the same as humans or livestock.

The findings across studies that GLP-1 production increases aborally is an interesting one. The distribution of intestinal L-cells and GLP-1 production is relatively consistent between species, despite the differences in digestion and physiology which exist between monogastric animals such as humans, foregut fermenters such as ruminants, and hindgut fermenters like the pig. Likewise, the actions of GLP-1 appear to be consistent, with appetite reduction, incretin effect, and effects on gastrointestinal motility similar between studied species. The stability of intestinal L-cell distribution and GLP-1 production between species is supportive of an important physiological role or roles. The preproglucagon gene evolved prior to the diversification of vertebrates more than 500 million years ago and has evolved more slowly in mammals than frogs and bony fish, suggesting it has a function(s) whose preservation favors survival or inheritance (Irwin, 2020). Whilst the glucose and appetite regulating functions of GLP-1 and glucagon are certainly important, it is possible that there are further, undiscovered or poorly understood roles of gut peptides, such as in regulation of reproduction.

There is an increasing body of evidence which suggests that GLP-1 can upregulate reproduction, including our own work in sheep, which demonstrated that administration of a GLP-1 agonist resulted in LH release. There are indications that this relationship is reciprocal, with GLP-1 production and the effect of GLP-1 varying depending on reproductive status i.e. stage of oestrous cycle, pregnancy or lactation. Glucose metabolism, including an altered incretin effect, is known to be different in women than men, as is gastric emptying time, which could be due to differences in GLP-1 function or

secretion (Handgraaf & Philippe, 2019). A spatial pattern could be seen, with expression tending to be higher in the distal gastrointestinal tract (distal ileum and colon) during the follicular phase than during the luteal phase whilst expression trended higher in the proximal intestine (jejunum and proximal ileum) during the luteal than the follicular phase. This spatial pattern may explain the difference between our observations and Johnson et al. (2017), who found that GLP-1 expression was greatest during pro-oestrus in the rat when compared to dioestrus or oestrus. That study, however, measured GLP-1 production in total, rather than comparisons within and between sites. Stage of oestrous cycle was not taken into account in a study in cows, which found that lactational status and reproductive maturity did not alter intestinal preproglucagon gene expression so it is uncertain whether expression varies across the oestrous cycle in ruminants (Connor et al., 2010). The mechanisms underlying how stage of oestrous cycle may influence GLP-1 production are unclear. It is possible that the relationship is indirect, with the decrease in feed intake associated with oestrus resulting in reduced GLP-1 release (Butera, 2010; Johnson et al., 2017). Conversely, feed intake may be reduced as a result of increased GLP-1 release. Sex steroids may alter the gut microbiome, which in turn could alter GLP-1 release (Handgraaf & Philippe, 2019). It is also possible that metabolism is altered in response to reproductive status, creating a more favourable environment for oestrus, conception and pregnancy. Oestrogen appears to have a direct effect on pancreatic α-cells, which secrete glucagon, and intestinal L-cells (Handgraaf et al., 2018). Oestrogen alters the Psck1/3 to Psck 2 ratio, altering post-translational processing of the preproglucagon gene to favor GLP-1 and GLP-2

production, rather than glucagon (Handgraaf et al., 2018). The role of other sex steroids, such as progesterone and testosterone, is less clear. However, it seems that for progesterone at least, any changes in GLP-1 production are a result of its moderation of the effects of oestrogen (Flock, Cao, Maziarz, & Drucker, 2013). Increased research into the effect of reproductive hormones on the function of the preproglucagon gene is important to allow better understanding of how GLP-1 based therapeutics differ in women, and how we may be able to manipulate and better manage these pathways in livestock species.

The distribution of GLP-1 expression in the ovine intestine in this study correlates with the pattern of GLP-2 and preproglucagon expression in cattle (Pezeshki et al., 2012). It seems most likely that intestinal-L cells are responsible for GLP-1 and GLP-2 production in the ruminant gastrointestinal tract, as in other species. Generally, the detection of ingesta in the intestinal lumen by nutrient-sensing receptors on the luminal surface of intestinal L-cells is believed to trigger GLP-1 secretion (Spreckley & Murphy, 2015). However, the high density of L-cells in the distal intestine is somewhat in contrast with the pattern of GLP-1 secretion typically occurring soon after eating, when ingesta would be entering the duodenum where L-cell numbers are relatively low (Holst, 2007). Neuronal or endocrine communication between different parts of the gastrointestinal tract may mediate GLP-1 release and effects. The vagal nerve appears to be involved in some GLP-1 signaling. Rats which have undergone vagal deafferentation are less sensitive to the anorectic effects of GLP-1 given via intra-peritoneal injection, but not when given into the portal vein (Spreckley & Murphy, 2015). However,

Nagell et al. (2006) found the same procedure had no effect on gastric motility in pigs. This may have been a result of the intravenous administration of GLP-1. These results suggest the vagus nerve is likely to be involved in mediating the effects of GLP-1, but other mechanisms are also important, such as direct interaction with the CNS or the enteric nervous system (Nagell et al., 2006). Another possibility is that the release and effects of GLP-1 release differ between the different parts of the gut-brain axis. GLP-1 release from the central nervous system and proximal intestine (eg duodenum, jejunum) in response to eating behaviour may be principally responsible for the short-term effects of appetite suppression, the incretin effect and gastric motility, whilst the larger, distal population of intestinal L-cells mediates longer-term effects, such as the upregulation of reproduction. There is some evidence for this, with Farilla et al. (2002) observing that chronic administration of GLP-1 has a trophic effect on pancreatic β-cells (Spreckley & Murphy, 2015) in one of the few studies on chronic effects of GLP-1 administration. Colonic L-cells are thought to have evolved under different evolutionary pressures than enteroendocrine cells elsewhere in the gastrointestinal tract and could have evolved to have a different physiological role (Reiman et al, 2008 and Moriya et al, 2014, in Paternoster and Falasaca, 2018). The slower passage of ingesta (and then faeces) through the distal intestine could be expected to result in more prolonged release of GLP-1 and the establishment of "baseline" concentrations of circulating GLP-1. More study is required to understand whether the stimuli of GLP-1 release differs in the distal intestine and the long-term effects of GLP-1.

Conclusion

The preproglucagon gene is expressed in the ovine intestine, with a pattern of distribution similar to that reported in other species despite differences in digestive physiology. It is unclear whether stage of oestrous cycle alters preproglucagon gene expression and GLP-1 production, however there is preliminary evidence that this may be the case.

Chapter 7: Exegesis

Research Chapter 7: Exegesis

There are strong endocrinological links between nutritional status and reproduction in animals. Our research builds on this understanding by providing insight into the relationship between nutrition and reproduction and how this may be mediated by GLP-1. We have achieved this by considering the effect of peripheral administration of a GLP-1 agonist and measuring the effect of an Exendin-4 the gonadotrophins LH and FSH in ewes. We also considered the effect of the oestrous cycle on results and investigated the expression of the preproglucagon gene, which encodes for the synthesis of GLP-1, in the ovine intestine. Our most notable findings are;

1) Systemic administration of a GLP-1 agonist, Exendin-4, stimulates LH secretion in the ewe.
2) The response of the hypothalamic-pituitary-gonadal axis to a GLP-1 agonist differs depending on stage of oestrous cycle in the ewe.
3) The preproglucagon gene is expressed in the gastrointestinal tract of the ewe

The relevance and importance of these findings are discussed below.

1) Systemic administration of a GLP-1 agonist stimulates LH secretion in the ewe.

A major finding from our preliminary study was that IV administration of 2mg of the GLP-1 agonist, Exendin-4, resulted in a significant and relatively rapid increase in LH secretion which could be detected in a peripheral blood sample in ewes in both the follicular and luteal phases of

the oestrous cycle. Based on these findings in the preliminary study, the main study focused on ewes in the follicular phase and confirmed the effect with larger numbers and the inclusion of a control group.

These results suggest that the hypothalamic-pituitary-gonadal axis can detect and respond to GLP-1 in peripheral circulation, such as that produced by the gastrointestinal tract. This is one of the few studies to consider the effect of peripheral GLP-1 on the HPG, as opposed to central administration (Arbabi et al., 2021; Beak et al., 1998; Outeirino-Iglesias et al., 2015). The potential for peripherally produced GLP-1 to affect the hypothalamus and/or pituitary is an important one, as the majority of GLP-1 is produced in the large intestine in most species. The sheep can now be included in the list of species that produce GLP-1 in the intestine as illustrated by our PCR study (*vide infra*).

It was beyond the scope of our study to determine the site of effect, however GnRH neurons in the hypothalamus remain a likely (final?) target. Our results would support GnRH neurons as the primary site of GLP-1 regulation of reproduction, as LH release has a direct, temporal relationship with GnRH secretion (Clarke & Cummins, 1982). The rapid increase in LH secretion seen in both our preliminary and main studies is thus likely to be a result of increased GnRH release. The observation in rodents that Exendin-4 resulted in a decrease in the amplitude of the LH surge, in contrast to GLP-1, is not corroborated by our findings. This difference may reflect the different reproductive physiology of the rat when compared to sheep and primates, although further

studies which compare Exendin-4 with GLP-1 would be useful. It is also possible that other neurons may be the primary target or an important intermediary in mediating the effect of GLP-1 on the reproductive axis. Hypothalamic RFRP3 neurons or the KNDy neurons of the ARC are candidates here. The role of KNDy neurons in integrating various peripheral signals to mediate the reproductive axis via kisspeptin release makes them a target worth investigating, particularly when considering the effect that the oestrous cycle had in our research.

Increased GnRH secretion can also increase FSH release. Although the results from the preliminary study suggested that Exendin-4 infusion resulted in increased plasma FSH concentrations, this was not corroborated in the main study, when a control group was included. Instead, it seems that the increase in FSH which occurred in both studies may have been a result of endogenous factors relating to the stage of oestrous cycle. However, a relationship between GLP-1 and FSH can not be excluded based on our research. Changes in plasma FSH concentrations are not as labile as LH and reflect the releasable pool in the pituitary, rather than being a direct reflection of GnRH release (Clarke et al., 2002). FSH also has a longer half-life than LH and its adenohypophyseal secretion is directly affected by gonadal hormones (e.g. oestrogen, inhibin and activin). These characteristics of FSH secretion may have obscured any effect of Exendin-4 infusion on FSH concentrations in our relatively short study.

Although a hypothalamic mechanism of action is suspected, it is also possible that peripheral Exendin-4 and GLP-1 act outside the blood-brain-barrier to regulate reproduction. This could be

manifest by a direct effect on gonadotrophs in the pituitary and/or on the gonads themselves. Interestingly, GLP-1R have been found on both pituitary and gonadal cells in rodents (Outeirino-Iglesias et al., 2015), which gives this concept some credence. Importantly, GnRH releasing terminals sit outside the blood-brain-barrier within the ME, so it is possible, even likely, that GLP-1 regulates GnRH secretion here rather than at the neuronal cell bodies. This concept is supported by the reports from Arbabi et al. (2021) where they found that microinjection of GLP-1 into the ME resulted in increased secretion of LH. Surprisingly, *in-situ* hybridization found that there was not expression of GLP-1R on GnRH neurons (Arbabi et al., 2021). This is somewhat in contrast to Farkas et al. (2016) who found that GLP-1 immunoreactive axons innervate GnRH neurons in the hypothalamus. The site of action for GLP-1 and its agonists remains unanswered, but may be directly on GnRH neurons, GnRH secretory terminals or via an intermediary cell type.

2) The response of the hypothalamic-pituitary-gonadal axis to a GLP-1 agonist differs depending on stage of oestrous cycle in the sheep.

An interesting finding from our preliminary study was the effect that stage of oestrous cycle had on LH and FSH release and response to Exendin-4.

Plasma concentrations of LH were higher in the follicular phase ewes before infusion and appeared to respond more quickly to Exendin-4 infusion when compared with ewes in the luteal phase. This

finding was used to inform the design of the main study, in which we used ewes exclusively in the follicular phase.

The increased baseline secretion of LH seen in the follicular phase makes biological sense, as baseline and pulsatile GnRH and LH secretion increases when the progesterone “block” of the luteal phase is not present. The apparent ability of Exendin-4 to overcome this restriction to some degree and result in increased LH secretion during the luteal phase (see Fig 3.3 in research chapter 3) is further supportive evidence of the role GLP-1 plays in regulation of reproduction. This response appeared to be delayed when compared to the follicular phase ewes. Pre-infusion plasma concentrations of LH were below detectable levels in all luteal phase ewes, so it is also possible that an increase in LH secretion occurred earlier than could be observed by the assays used in the study.

The difference in response seen between the stages of oestrous cycle would suggest that the response of the HPG axis to GLP-1 varies depending on steroid status of the ewe. Whether this altered response is due to increased sensitivity to GLP-1 e.g. via increased GLP-1R expression or reflects a greater capacity of gonadotrophs to respond due to increased production and stores of LH during the follicular phase is unknown. A study in rats found that GLP-1R expression in the HPG axis varies across the oestrous cycle (Outeirino-Iglesias et al., 2015), however rodent reproduction is sufficiently different from ruminants and humans that direct extrapolation of results has inherent flaws. A molecular study of GLP1-R expression and colocalization on GnRH neurons, neurosecretory terminals

and pituitary gonadotrophs using qPCR or *in-situ* hybridization in sheep would be an informative way to explore this question.

The effect of oestrous cycle on our results in the preliminary study also highlights the importance of hormonal status when designing and interpreting studies involving the reproductive tract. The use of gonadectomized animals or those in which the hypothalamus has been impaired can be a convenient method of negating this issue, however is problematic. The results obtained from such studies may not reflect what occurs physiologically, as the removal of pulsatile GnRH and steroid feedback mechanisms alters gonadotroph production and secretion. Differences in steroid status may also explain some of the discrepancies in results which can occur between studies.

3) The preproglucagon gene is expressed in the gastrointestinal tract of the ewe

We have confirmed that the preproglucagon gene is expressed in the ovine gastrointestinal tract. To our knowledge this is the first published report to do so. The use of qPCR on multiple sites in the gastrointestinal tract allows for some interesting observations. Firstly, it demonstrates the spatial pattern of preproglucagon gene expression (and, indirectly, intestinal L cell distribution) in the sheep, with expression in the large intestine markedly higher than the forestomachs and proximal intestinal tract. Unsurprisingly, the pattern of distribution in sheep is similar to that of cattle, with *GCG* expression very low in the foregut (reticulum, rumen, omasum and abomasum) and greatest in the

large intestine (colon and rectum) (Connor et al., 2010). It is of interest, however, that the pattern seen in in ruminants is not dissimilar from monogastric species, despite the considerable differences in anatomy, digestive physiology and feeding behaviour. This suggests GLP-1 production and activity have changed very little during mammalian evolution, a finding supported by genomic sequencing and phylogenetic studies (Irwin, 2020). Such findings are consistent with an important biological role or roles of GLP-1 (Irwin, 2001).

The biological importance of GLP-1 may be primarily due to its role in appetite and glucose regulation, however other functions, such as regulation of reproduction, need to be considered. Indeed, the ability of a gut peptide to upregulate reproduction during favorable nutritional conditions would be expected to confer an evolutionary advantage that could be readily passed on to offspring.

Ruminant digestive physiology differs significantly from those of monogastric animals where fermentative digestion occurs in the foregut, ingestion activity (grazing) is typically prolonged, and release of ingesta into the intestine from the stomach is related to rumination, rather than ingestion, and tends to also be prolonged. The observation that the pattern of GLP-1 release and preproglucagon gene expression is similar between these two groups is fascinating and highlights the value in performing such studies across species. That L-cell distribution seems to be more strongly related to anatomy than to physiological differences between species, such as site of fermentation, is itself a valuable observation in better understanding intestinal L-cell function.

Whether preproglucagon gene expression varies during the oestrous cycle could not be determined from our studies due to a small sample size. There is growing evidence that a reciprocal relationship exists between the digestive tract and reproduction (Handgraaf et al., 2018; Johnson et al., 2017); but how this relationship is mediated is uncertain. Steroids such as oestrogen appear to play a role in moderating GLP-1 secretion (Handgraaf & Philippe, 2019), but the potential role or effect on gut peptides and other appetite regulating hormones is not well explored. Interestingly, the effect of oestrous cycle may vary depending on site. Expression tended to be increased in the distal GIT during the follicular phase but was decreased in the proximal GIT when compared with expression during the luteal phase. A more extensive study in animals of known oestrous status and under controlled feeding conditions could be informative, especially if matched with hypothalamic studies, as mentioned above and plasma concentrations of endogenous GLP-1. Our results suggest that multiple GIT samples are required to make genuine comparisons, rather than single site or total concentrations such as used by Johnson et al. (2017) in a rodent study.

Since this research project was first conceived there has been ongoing research into the link between nutrition and reproduction. Our finding that peripheral GLP-1 is likely to upregulate the reproductive axis provides an important link in understanding how this relationship is mediated. A better understanding of how GLP-1 and other appetite-related peptides can modify reproduction should allow optimal management of livestock for fertility, particularly in times of limited feed availability,

such as during drought. It also identifies a potential point of fertility failure that may be able to be targeted therapeutically.

Importantly, the use of GLP-1 agonists to manage weight loss and type 2 diabetes in humans has become more widespread. The GLP-1 agonist semaglutide (Ozempic ®, Novo Nordisk Pharmaceuticals Pty. Ltd, Baulkham Hills NSW) has received considerable recent media attention due to its efficacy as a weight loss drug. Understanding how GLP-1 interacts with the reproductive axis is important in understanding why there may be treatment differences between men and women, any unintentional side effects and other possible therapeutic targets e.g. poly-cystic ovarian syndrome.

Conclusion

We have demonstrated that systemic administration of a GLP-1 agonist can stimulate LH secretion in female sheep and suggesting gut-to-brain signaling is a realistic phenomenon, in terms of reproductive function. The response varies during the oestrous cycle, being more profound in the follicular phase of the cycle.

We have also confirmed that the preproglucagon (*GCG*) gene is expressed in the ovine gastrointestinal tract. Our preliminary observation that GLP-1 expression is greatest in the distal regions of the gastrointestinal tract should prompt further studies on the expression of *GCG* expression, how this relates to endogenous GLP-1 production and possible variation in each across the oestrous cycle.

These findings contribute to the scientific knowledge base regarding nutritional regulation of reproduction, particularly where it involves GLP-1.

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